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High content cellular immune profiling reveals differences between rhesus monkeys and men

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Summary

A better understanding of similarities and differences in the composition of the cellular immune system in non-human primates (NHPs) compared with human subjects will improve the interpretation of preclinical studies. It will also aid in addressing the usefulness of NHPs as subjects for studying chronic diseases, vaccine development and immune reconstitution. We employed high content colour flow cytometry and analysed simultaneously the expression of CD3, CD4, CD8α, CD8β, CD16/CD56, CD45RA, CCR7, CD27, CD28, CD107a and the interleukin-7 receptor α-chain (IL-7Rα) in peripheral blood mononuclear cells (PBMCs) of 27 rhesus macaques and 16 healthy human subjects. Regulatory T cells (Tregs) were identified using anti-CD3, -CD4, -CD25, -FoxP3, and -IL-7Rα monoclonal antibodies. Responsiveness to IL-7 was gauged in a signal transducer and activation of transcription 5 (STAT-5) phosphorylation assay. Human and NHP PBMCs showed a similar T-cell composition pattern with some remarkable differences. Similarities: human and NHP $CD4^+$ and $CD8^+$ cells showed a similar STAT-5 phosphorylation pattern in response to IL-7. Multicolour flow cytometric analysis identified a $CD4^+$ $CD8\alpha\alpha^+$ $CD8\alpha\beta^+$ T-cell population in NHPs as well as in human subjects that expressed the degranulation marker CD107a and may represent a unique $CD4^+$ T-cell subset endowed with cytotoxic capacity. Differences: we identified in PBMCs from NHPs a higher proportion (5·16% in $CD3^+$ T cells) of $CD8\alpha\alpha^+$ T cells when compared with human donors (1·22% in $CD3^+$ T cells). NHP $CD8\alpha\alpha^+$ T cells produced tumour necrosis factor-α / interferon-γ (TNF-α/IFN-γ) or TNF-α, whereas human $CD8\alpha\alpha^+$ T cells produced simultaneously TNF-α/IFN-γ and IL-2. A minor percentage of human $CD8^+$ T cells expressed $CD25^{bright}$ and FoxP3 (0·01%). In contrast, 0·07% of NHP $CD8^+$ T cells exhibited the $CD25^{bright}$ $FoxP3^+$ phenotype. PBMCs from NHPs showed less IL-7Rα-positive events in all T-cell subsets including $CD4^+$ Tregs (median 5%) as compared with human (median 12%). The data visualize commonalities and differences in immune cell subsets in humans and NHPs, most of them in long-lived memory cells and cells with suppressive functions. This provides a matrix to assess future efforts to study diseases and vaccines in NHPs.

Keywords: flow cytometry; immune markers; immune profiling; non-human primate model; T cells; vaccination

Introduction

Non-human primates (NHPs) provide an indispensable model to study human diseases, including chronic infections and human immunodeficiency virus and tuberculosis vaccine development.[1,2] They have been instrumental in the study of aging and immune reconstitution.[3–6] Despite general differences in T-cell immunology between

species, other factors play an important role in gauging immune responses. Animals live in a protected environment and are not exposed to the same pathogens that affect humans. This may impact on the breadth of the cellular immune repertoire and on the maintenance of immune cell memory.[7–12] Other observations underline the need to study the differences between human and NHP immune responses: a humanized anti-CD28 monoclonal superagonist antibody caused severe side-effects in a phase 1 clinical trial;[13] it induced a delayed and sustained Ca^{2+}-influx in human $CD4^+$ T cells, but not in $CD4^+$ T cells from NHPs.[14] Any experimental study of cellular, adaptive immune responses addresses also T-cell homeostasis, the active and dynamic process by which immune cells mature traffic and produce cytokines upon activation. Key elements of the analysis of adaptive cellular immune responses are (i) T-cell subsets (CD4/CD8, $CD8\alpha\alpha^+$ memory T cells) in concert with differentiation and homing markers (CD45RA, CCR7, CD28, CD27, CD62L),[15] cytotoxicity (measure of CD107a) and cytokine production (polyfunctionality);[16] (ii) regulatory T cells (Tregs);[17] and (iii) the response to interleukin-7 (IL-7), a key cytokine for T-cell survival, homeostasis and T-cell memory.[18] T-cell compartment composition and phenotype has been studied previously in rhesus macaques[19,20] with a limited panel of immune markers. Different combinations of immune markers were used in these studies to define memory and effector T-cell compartments in rhesus monkey.[21] To our knowledge, the current report analyses for the first time the simultaneous expression of CD45RA, CCR7, CD27 and CD28 in T-cell subsets in healthy rhesus monkeys. We took advantage of a high-content, multicolour flow cytometry to assess the distribution of immune cells in peripheral blood mononuclear cells (PBMCs) from female rhesus monkeys (defined by expression of CD45RA, CCR7, CD28, CD27, CD107a, IL-7 receptor α-chain), to compare cytokine [IL-2, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α)] production in T-cell subsets, IL-7-induced signal transducer and activator of transcription 5 (STAT-5) phosphorylation, and Treg frequencies.

Materials and methods

Blood samples

Peripheral blood was obtained from 16 healthy human donors (19–66 years, median 31 years) from the Blood Bank (Ethical Permit DNR 00-097). Peripheral blood was obtained from 27 female rhesus macaques (*Macaca mulatta*) of Chinese origin with an age range between 3 and 4 years housed in the Astrid Fagraeus laboratory at the Swedish Institute for Infectious Disease Control. Housing of the animals and care procedures were in compliance with the provisions and general guidelines of the Swedish Animal Welfare Agency, the Local Ethical Committee responsible for Animal Experiments approved all procedures (protocol DNR238/2006-54). The PBMCs were isolated from freshly obtained, heparinized peripheral blood by Ficoll–Hypaque density gradient centrifugation.

Multicolour flow cytometric analysis

Immune marker analysis was performed on freshly isolated PBMCs by a standard Ficoll procedure from heparinized blood samples. Cells (1×10^6) were incubated at 4°C for 15 min with the following antibodies: phycoerythrin (PE) -Cy7-conjugated anti-CCR7 (3D12), peridinin chlorophyll protein (PerCP) -conjugated anti-CD3 (SP-34-2), PE-conjugated anti-CD16 (3G8), PE-conjugated anti-CD56 (B159), allophycocyanin (APC) -Cy7-conjugated anti-CD8α chain (SK1), Amcyan-conjugated anti-CD28 (CD28.2), purchased from BD Biosciences (Stockholm, Sweden), APC-Alexa Fluor 700-conjugated anti-CD107a (H4A3), APC-conjugated anti-IL-7 receptor α-chain (IL-7Rα; R34.34), PE-Texas Red-conjugated anti-CD45RA (2H4), fluorescein isothiocyanate (FITC) -conjugated anti-CD8β chain (2ST8.5H7), purchased from Beckman Coulter (Marseille, France), and Pacific Blue-conjugated anti-CD4 (S3.5) purchased from Caltag Laboratories (Burlingame, CA). The lymphocytes were then washed in phosphate-buffered saline (PBS) with 0·1% fetal bovine serum, and incubated at 4° for 15 min with the anti-CD27 (1A4CD27) antibody (Beckman Coulter) labelled with Pacific Orange using the Zenon Pacific Orange Mouse IgG1 Labeling Kit obtained from Invitrogen (Stockholm, Sweden). Human samples were processed the same day, and NHP samples were processed on a different occasion, but also the same day. The median fluorescence intensity (MFI) of IL-7Rα expression therefore allows a comparison of the intensity of IL-7Rα expression on T cells within each species but not between humans and NHPs. Data acquisition was performed using a FACSAria Flow cytometer (BD Biosciences) and results were analysed with FlowJo software (Tree Star Inc., Ashland, OR).

Intracellular cytokine staining

Cytokine production was analysed in frozen PBMCs, which were thawed, rested overnight and stimulated for 6 hr in the presence of brefeldin A (10 mg/ml) purchased from Sigma-Aldrich (Sweden AB, Stockholm, Sweden) either with medium: RPMI-1640 containing L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (10 mg/ml), 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen), or medium and phorbol 12-myristate 13-acetate (PMA)/ionomycin (25 ng/ml and 1 mg/ml, respectively; Sigma-Aldrich). Cells were then washed in PBS,

and stained with cell surface marker antibodies: Pacific Blue-conjugated anti-CD3 (SP34-2), PerCP-Cy5.5 conjugated anti-CD4 (L200; BD Biosciences), APC-Cy7-conjugated anti-CD8α chain (SK1), and FITC-conjugated anti-CD8β chain (2ST8.5H7), in the presence of the live/dead fixable dead cell marker (Aqua LIVE/DEAD; Invitrogen), for 30 min at 4°. After washing with PBS, cells were fixed and permeabilized using the IntraPrep Fix/Perm Kit (Beckman Coulter) and incubated with antibodies specific for intracellular cytokines for 30 min at 4°: PE-conjugated anti-IL-2 (MQ1-17H12), PE-Cy7-conjugated anti-IFN-γ (B27), and APC-conjugated anti-TNF-α, all obtained from BD Biosciences. Cells were analysed using a BD FACSCanto flow cytometer (BD Biosciences) and data analysis was performed using FlowJo software.

T helper type 17 induction and detection of IL-17-producing cells

Human and NHP frozen PBMCs were thawed, rested overnight and distributed into 96-well plates ($0{\cdot}4 \times 10^6$ cells/well) coated with 50 μl anti-CD3 (OKT3, 1 μg/ml) and anti-CD28 (CD28.2; Beckman Coulter, 1 μg/ml) antibodies. Cells were cultured in AIM-V/Dulbecco's modified Eagle's medium (Gibco, Invitrogen) containing 1% heat-inactivated human serum (Biowest, Nuaillé, France), either (i) in medium alone, (ii) in the presence of human IL-23 (10 ng/ml) (Humanzyme, Chicago, IL) or (iii) in a combination of IL-23 with human IL1-β (10 ng/ml) (R&D Systems, Abingdon, UK). After 6 days, cells were stimulated with PMA/ionomycin for 6 hr, and IL-17, IFN-γ and TNF-α production was detected in CD4$^+$, CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ T cells as described above, using a PE-conjugated anti-IL-17 antibody (eBio64DEC17) purchased from eBioscience (San Diego, CA) simultaneously with PE-Cy7-conjugated anti-IFN-γ (B27) and APC-conjugated anti-TNF-α antibodies.

IL-7-induced STAT-5 phosphorylation assay

Constitutive and IL-7-induced phosphorylated STAT-5 (P-STAT-5) expression was evaluated in frozen PBMCs as described previously.[71] Briefly, overnight starved, thawed PBMCs were incubated with recombinant human IL-7 (rhIL-7; 100 ng for 10^5 cells, provided by Dr Michel Morre, Cytheris, Issy-les-Moulineaux, France) for 15 min at 37°. The cells were then incubated for 15 min at 4° with the following cell surface antibodies: APC-conjugated anti-CD4 (SK3; BD Biosciences), and APC-Cy7-conjugated anti-CD8α chain, and immediately after fixed with 2% paraformaldehyde. The cells were washed with Stain Buffer (BD Biosciences) and permeabilized with 90% methanol for 30 min on ice, followed by two washes with Stain Buffer. The cells were incubated with Alexa-Fluor 488-conjugated anti-P-STAT-5a antibody (Y694) (BD Biosciences) for 1 hr at room temperature and analysed immediately using a FACSAria flow cytometer and data analysis was performed using FlowJo software. Because of the fixation procedure, we could not include the anti-CD3 monoclonal antibody as it did not exhibit sufficient stability in the fixation procedure required for intracellular staining, so the data are obtained by gating on CD8$^+$ and CD4$^+$ cells for STAT-5 phosphorylation analysis. The anti-CD8β chain antibody could not be used in this panel (also because of the fixation procedure). The CD8$^+$ subset encompasses therefore the CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ cell subsets. Human IL-7 shows similar activity to NHP IL-7 (personal communication, Dr Michel Morre, Cytheris, Issy-les-Moulineaux, France).

Regulatory T cells

Frozen PBMCs were thawed and incubated at 4° for 15 min with the following antibodies: PerCP-conjugated anti-CD3 (SP34-2), PerCP Cy5.5-conjugated anti-CD4 (L200), APC-Cy7-conjugated anti-CD8α chain (SK1), APC-conjugated anti-IL-7Rα (R34.34), PE-Cy7-conjugated anti-CD25 (2A3; BD Biosciences). The PBMCs were then washed with Stain Buffer (BD Biosciences) and fixed with FOXP3 Fix/Perm Buffer (BioLegend, San Diego, CA) at room temperature for 20 min followed by one washing with Stain Buffer and one washing with FOXP3 Perm Buffer (BioLegend). The PBMCs were resuspended in FOXP3 Perm Buffer and incubated at room temperature for 15 min. After washing, cells were resuspended in FOXP3 Perm Buffer and incubated at room temperature for 30 min with the Alexa-Fluor 488-conjugated anti-FoxP3 (259D) antibody purchased from BioLegend. Analysis was performed using a FACSAria flow cytometer and data were analysed using FlowJo software. CD8β-expressing cells could not be measured because the monoclonal antibody anti-CD8β chain did now exhibit sufficient stability in the fixation procedure required for FoxP3 protein analysis.

Statistics

Data are presented as median ± SD, and *P*-values were derived using a Mann–Whitney *U*-test.

Results

NHPs and humans share a similar T-cell compartment

The phenotype of the T-cell compartment in the peripheral blood of 16 healthy human donors (HDs) and 27 rhesus monkeys was assessed by multicolour flow cytometric analysis. CD3$^-$ lymphocytes, which express CD56 and CD16, identify natural killer (NK) cells in humans.

CD56 identifies mainly monocytes and $CD16^+$ NK cells in rhesus macaques.[22] T lymphocytes were determined by CD3 expression and after exclusion of $CD16^+$ and $CD56^+$ cells (gating strategy see Fig. 1a). The (co)expression of CD4, CD8α and CD8β in the T-cell ($CD56^-$ $CD16^-$ $CD3^+$) compartment was determined in HDs and NHPs. CD8$\alpha\beta^+$ T cells and CD8$\alpha\alpha^+$ T cells represented 23·8% and 1·2% in HDs, and 28% and 5·2% in NHPs. In PBMCs from HDs and NHPs, $\gamma\delta$ T-cell receptor $(TCR)^{+/-}$ cells exhibited the CD8$\alpha\alpha^{+/-}$ phenotype. Yet the majority (> 70%) of CD8$\alpha\alpha^{+/-}$ T cells were present in the TCR-$\alpha\beta$ T-cell compartment (data not shown). $CD4^+$ T cells represented the prevalent T-cell subset: 74·3% and 63·6% of T cells in HDs and NHPs, respectively. Two other less frequent cell subsets could be identified: $CD4^+$ T cells expressing either the CD8$\alpha\alpha$ homodimer or the CD8$\alpha\beta$ heterodimer (0·2% and 0·1% in HDs; 1·3% and 1·4% in NHPs) (see Fig. 1b). CD8$\alpha\alpha^+$, $CD4^+$ CD8$\alpha\alpha^+$ and $CD4^+$ CD8$\alpha\beta^+$ T cells showed a statistically higher frequency in NHPs than in HDs.

Four functional T-cell compartments are defined in humans by the expression of CD45RA and CCR7: precursor ($CD45RA^+$ $CCR7^+$), central memory ($CD45RA^-$ $CCR7^+$), effector memory ($CD45RA^-$ $CCR7^-$) and differentiated effector ($CD45RA^+$ $CCR7^-$) T-cell subsets.[15,23] The distribution of the T-cell subsets defined by CD45RA and CCR7 expression within the different T-cell populations was statistically different in PBMCs between HDs and NHPs (Table 1).

We assessed the CD28 and/or CD27 expression within the CD45RA/CCR7 subsets. The median value of the expression frequency of $CD45RA^{+/-}$ $CCR7^{+/-}$ $CD28^{+/-}$ $CD27^{+/-}$ subsets in the parental T-cell population from the PBMC of HDs and NHPs is displayed as heat-maps (Fig. 2). In PBMCs from HDs, precursor, effector memory and central memory CD8$\alpha\beta^+$ T-cells co-expressed CD28 and CD27 ($CD28^-$ $CD27^+$ and $CD28^+$ $CD27^-$ subsets were also found). In contrast, differentiated effector CD8$\alpha\beta^+$ T cells were enriched in cells expressing only CD27. In NHPs, $CD45RA^+$ $CCR7^+$ and $CD45RA^+$ $CCR7^-$ cells represented the dominant T-cell subsets in the CD8$\alpha\beta^+$ T-cell compartment, and the expression of CD28 and CD27 differed from that by HDs within these T-cell compartments. In NHPs, CD8$\alpha\beta^+$ T cells expressed predominantly either CD28, or only CD27. We observed also an enrichment of $CD28^-$ $CD27^-$ (and a parallel decrease of $CD28^+$ $CD27^+$) T cells in PBMCs from NHPs compared with HDs.

The CD8$\alpha\alpha^+$ T-cell subset displayed a different profile as compared to CD8$\alpha\beta^+$ T cells. In HDs, CD8$\alpha\alpha^+$ T cells were enriched in differentiated T-cells (particularly $CD45RA^{+/-}$ $CCR7^-$) as compared to CD8$\alpha\beta^+$ T cells. Effector memory CD8$\alpha\alpha^+$ T cells expressed CD28 alone or in combination with CD27, and differentiated CD8$\alpha\alpha^+$

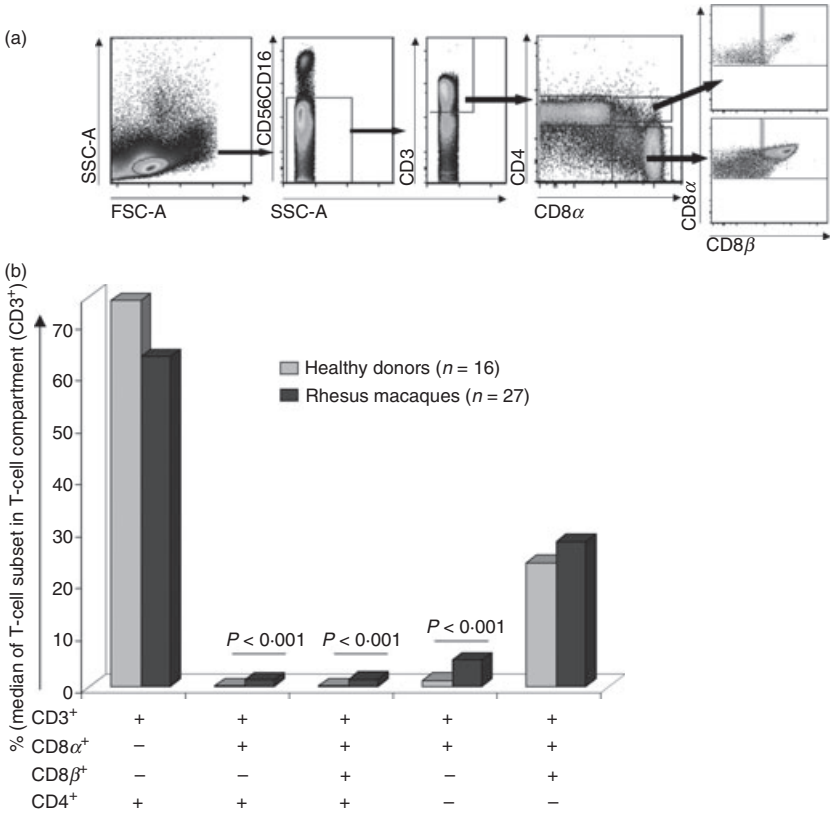

Figure 1. Identification of T-cell subsets. (a) Gating strategy to identify T cells, based on expression of CD3, CD8α, CD8β and CD4. (b) T-cell frequencies. CD8$\alpha\alpha^+$, $CD4^+$ CD8$\alpha\alpha^+$ and $CD4^+$ CD8$\alpha\beta^+$ T-cell frequencies were statistically higher in non-human primates. $P < 0{\cdot}001$ (Mann–Whitney *U*-test).

	CD45RA$^+$ CCR7$^+$	CD45RA$^+$ CCR7$^+$	CD45RA$^+$ CCR7$^+$	CD45RA$^+$ CCR7$^+$
CD8$\alpha\beta^+$				
Healthy donors	47·6 ± 10·7**	29 ± 7·1*	10·3 ± 6·8**	13·1 ± 6·8**
Rhesus macaques	*70 ± 9·2*	*25·7 ± 9·3*	*2·7 ± 2·1*	*1·5 ± 1·3*
CD8$\alpha\alpha^+$				
Healthy donors	19·4 ± 9·1**	27·8 ± 10·1**	19·4 ± 8·5**	33·4 ± 10·2**
Rhesus macaques	*46·3 ± 7·8*	*49·7 ± 7·7*	*1·6 ± 1·6*	*2·3 ± 2·4*
CD4$^+$				
Healthy donors	35·4 ± 7·9**	20 ± 5·8**	23·9 ± 5·3**	20·7 ± 6·5**
Rhesus macaques	*73·4 ± 9·7*	*12·9 ± 5·6*	*11 ± 7·6*	*2·5 ± 2·2*
CD4$^+$ CD8$\alpha\alpha^+$				
Healthy donors	24·8 ± 6·7**	19·6 ± 6·9*	26·7 ± 7·7**	28·8 ± 7·7**
Rhesus macaques	*59·2 ± 10·6*	*28·3 ± 9·2*	*7·4 ± 6·2*	*5 ± 3·4*
CD4$^+$ CD8$\alpha\beta^+$				
Healthy donors	39·1 ± 14·1**	17·1 ± 10·3*	27·2 ± 13·4**	16·5 ± 7·1**
Rhesus macaques	*83·7 ± 6·5*	*10·8 ± 5·2**	*4·8 ± 3·3*	*0·6 ± 0·6*

Table 1. T-cell compartments CD45RA/CCR7 expression in healthy human donors and non-human primates

Healthy donors $n = 16$, rhesus macaques $n = 27$. (%) Mean values and standard deviation. $^{**}P < 0{\cdot}0001$, $^{*}P < 0{\cdot}05$ (Mann–Whitney U-test).

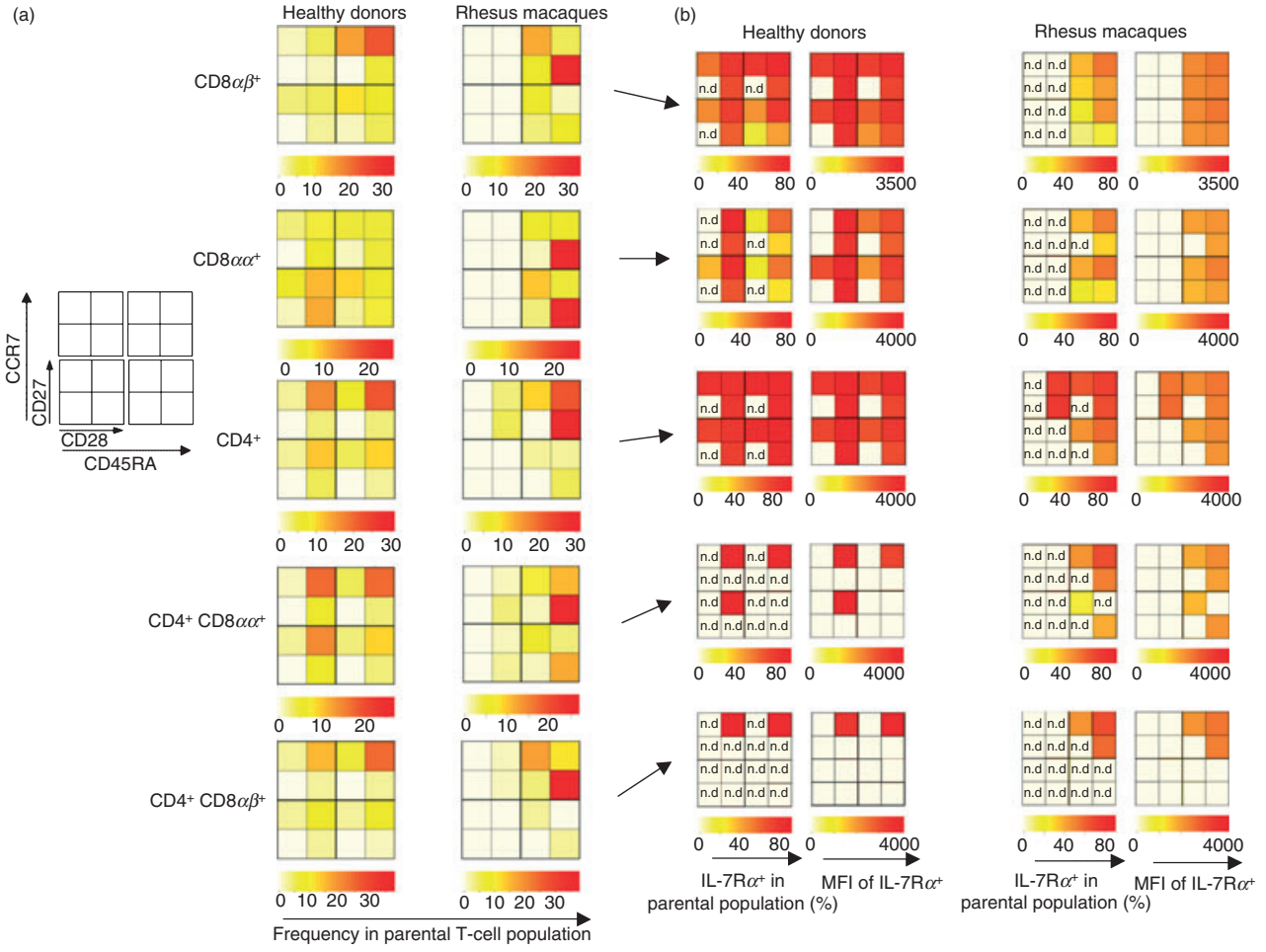

Figure 2. Overview of T-cell subsets defined by CD45RA/CCR7 and CD27/CD28 expression using heat-map analysis. (a) Frequency of immune cell subsets in human donors and non-human primates. (b) Interleukin-7 receptor α (IL-7Rα) expression and relative IL-7Rα density [as measured by mean fluorescence intensity (MFI)] in these T-cell subsets. The percentage of IL-7Rα (and MFI) in T-cell subsets displaying low number of events (< 100 events) was not determined (n.d.) for quality control reasons.

T cells CD27 or CD28. In NHPs, CD8αα$^+$ T cells displayed either a CD45RA$^+$ CCR7$^+$ or a CD45RA$^+$ CCR7$^-$ profile. Most of the CD45RA$^+$ CCR7$^\pm$ CD8αα$^+$ T cells stained positive only for CD28.

CD4$^+$ T cells were observed within the four CD45RA$^{+/-}$ CCR7$^{+/-}$ compartments in HDs, whereas 75·5% of CD4$^{+/-}$ T cells from NHPs stained positive for CD45RA$^+$ CCR7$^+$. Similar to the phenotype of CD8$^+$ T cells, NHP CD4$^+$ T cells were enriched in cells expressing only CD28 and not CD27. Interestingly, CD4$^{+/-}$ CD8αβ$^{+/-}$ T cells displayed a phenotype, based on CD45RA and CCR7 expression, comparable (not statistically different) to CD4$^\pm$ T cells in PBMCs from HDs. Of note, CD4$^+$ CD8αα$^+$ and CD4$^+$ CD8αβ$^+$ T cells represented the only immune cell subsets that stained positive for CD107a$^+$ (particularly in CD45RA$^+$ CCR7 cells expressing CD28 and or CD27): 5·5% and 3·7% of total CD4$^+$ CD8αα$^+$ and CD4$^+$ CD8αβ$^+$ T cells in HDs, and 1·3% and 1·7% in NHPs (data not shown).

In HDs, most CD8αβ$^+$ T cells and approximately 50% of CD8αα$^+$ T cells expressed the IL-7Rα. CD4$^+$ T cells and CD4$^+$ CD8αα$^+$ CD8αβ$^+$ T cells showed an increased frequency of IL-7Rα$^+$ T cells and higher levels of IL-7Rα expression/cell (measured by MFI) compared with CD8$^+$ T cells. The PBMCs obtained from NHPs showed a similar trend for IL-7Rα expression to HDs: more CD4$^+$ T cells expressed more IL-7Rα compared with the CD8$^+$ T-cell subsets, but the frequency of IL-7Rα$^+$ in all T-cell subsets was decreased in PBMCs obtained from NHPs compared with the frequency observed in HDs (e.g. in 86% of CD4$^+$ T cells in HDs and 67% in NHPs were IL-7Rα$^+$, Fig. 2b).

Differential cytokine production in T cells from humans compared with NHPs

The cytokine profile of CD4$^+$, CD4$^+$ CD8$^+$, CD8αα$^+$, CD8αβ$^+$ and CD4$^-$ CD8$^-$ T cells upon PMA/ionomycin stimulation (used to induce maximal cytokine production) in NHPs (n = 27) and HDs (n = 5) was assessed. The frequency of different T-cell subsets in the medium control and upon PMA/ionomycin stimulation (Fig. 3a) was similar in PBMCs from NHPs. In HDs, the frequency of CD4$^-$ CD8$^-$ T cells upon PMA/ionomycin stimulation was increased (from 3·6% to 10%) as a result of the down-regulation of CD4 and CD8 co-receptors in the CD4$^+$ and CD8αβ$^+$ T-cell subsets[24] (and concomitant decreased frequency of those subsets upon PMA/ionomycin stimulation as seen in some HDs). In PBMCS from NHPs and from HDs, CD4$^+$ and CD8αα$^+$ T cells showed similar frequencies of cytokine-producing cells in response to PMA/ionomycin stimulation. In contrast, higher frequencies of cytokine-producing cells were detected in the CD8αβ$^+$, CD4$^+$ CD8$^+$, and CD4$^-$ CD8$^-$ T-cell compartments in HDs compared with NHPs (Fig. 3b). CD4$^-$ CD8$^-$ T cells were sorted by fluorescence-activated cell sorting, followed by intracellular staining with anti-cytokine (IL-2, TNF-α, IFN-γ), -CD4 and -CD8 monoclonal antibodies to decipher whether the increased frequency of cytokine producing CD4$^-$ CD8$^-$ T

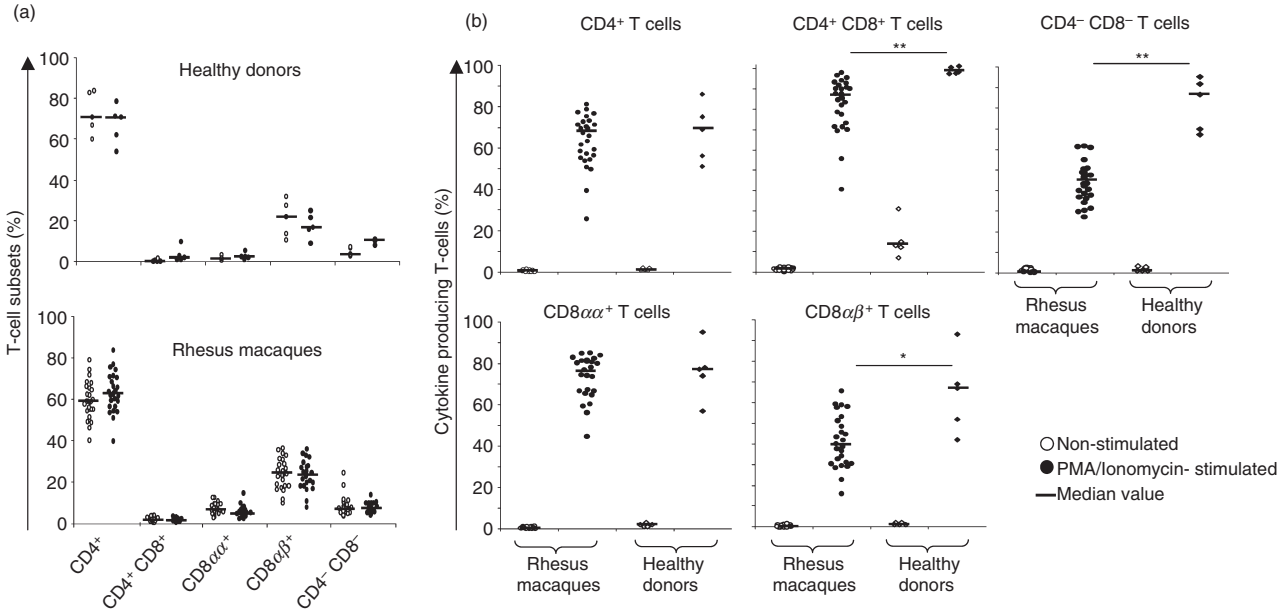

Figure 3. Cytokine producing T-cell subsets in human donors and non-human primates. (a) Presence of immune cell subsets defined by CD3, CD4, CD8α and CD8β expression with no stimulation (medium control) and phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation. (b) Percentage of cytokine producing T cells. $^{**}P < 0{\cdot}001$, $^{*}P < 0{\cdot}05$ (Mann–Whitney U-test).

cells after PMA/ionomycin stimulation in PBMCs from HDs as compared to NHPs was the result of 'bona fide' $CD4^-$ $CD8^-$ T cells or to T cells that down-regulated the cell surface expression of the CD4 or CD8 co-receptors. The $CD4^-$ $CD8^-$ T cells from HDs that do not express (at the cell surface or intracellularly) CD4 or CD8 showed a higher frequency of cytokine-producing cells than the NHPs $CD4^-$ $CD8^-$ T cells (data not shown).

The production of IL-2, TNF-α and IFN-γ was measured simultaneously on the single cell level to assess the presence of polyfunctional T cells. The profile of two representative PBMC samples from monkeys and from two HDs is shown in Fig. 4. In NHPs, $CD4^+$ T cells produced TNF-α and IL-2, either in combination or alone, $CD8\alpha\beta^+$ T cells produced mainly IFN-γ and TNF-α, either in combination or alone, and to a lesser extent IL-2. The $CD8\alpha\alpha^+$ T-cell subset showed a cytokine production profile very similar to that of the $CD8\alpha\beta^+$ T-cell subset. $CD4^+$ $CD8^+$ T cells displayed a polyfunctional profile (the vast majority of $CD4^+$ $CD8^+$ T cells produced two or three cytokines simultaneously). $CD4^-$ $CD8^-$ T cells displayed a profile similar to $CD4^+$ T cells, they produced IL-2 and TNF-α, but also IL-2 or TNF-α alone. The cytokine profile in the different T-cell compartments from HDs was very similar to the profile identified in NHPs, but they exhibited a higher frequency of polyfunctional T cells (e.g. 18·8% of $CD8\alpha\beta^+$ T cells in NHPs produced three cytokines compared with 27·2% in HDs). To further characterize the different T-cell subsets, we assessed the presence of $IL\text{-}17^+$ producing T cells. The PBMCs from four HDs were either cultured without cytokines, or in Th17 differentiation conditions (in the presence of IL-23 either alone or in combination with IL-1β). The combination of IL-23 and IL-1β was found to induce the highest frequency of $IL\text{-}17^+$ producing cells. $CD4^+$ $CD8^+$ T cells showed, after PMA/ionomycin stimulation, an enrichment in $IL\text{-}17^+$ producing cells compared with $CD4^+$ T cells (Fig. S1). In the presence of IL-23 and IL-1β, IL-17 production was detected in 20% (median value) of $CD4^+$ $CD8^+$ T cells, and in 10% of $CD4^+$ T cells. Interleukin-17 was produced in combination with TNF-α in $CD4^+$ $CD8^+$ and $CD4^+$ T cells and to a lesser extent also with IFN-γ. Higher frequencies of $IL\text{-}17^+$ producing cells were detected in $CD8\alpha\alpha^+$ than in $CD8\alpha\beta^+$ T cells. The NHP PBMCs from five animals were cultured using identical conditions, yet we could not study the nature of $IL\text{-}17^+$ T cells because of the low number of IL-17-positive events.

IL-7-induced STAT-5 phosphorylation in NHPs and humans

The binding of IL-7 to the IL-7Rα induces the activation by phosphorylation of the transcription factor STAT-5. IL-7 and IL-7 T-cell responsiveness play a pivotal role in T-cell homeostasis and in immune formation.[18] A STAT-5 phosphorylation assay was used to gauge IL-7 responsiveness in $CD4^+$ and $CD8^+$ cells. The increase of the percentage of P-STAT-5 cells, and an example of constitutive P-STAT-5 and IL-7-induced P-STAT-5, in HD and NHP are shown in Fig. 5(a,b). In NHPs, (n = 15) 84·4 ± 10·8% and 60·6 ± 12% of $CD4^+$ and $CD8^+$ cells showed an increase of P-STAT-5 cells in response to IL-7 stimulation. Similar numbers were obtained in PBMCs from HDs (n = 10): 87·6 ± 7·6% and 62·3 ± 15·4% in $CD4^+$ and $CD8^+$ cells, respectively. $CD4^+$ and $CD8^+$ subsets showed comparable responses to IL-7 stimulation

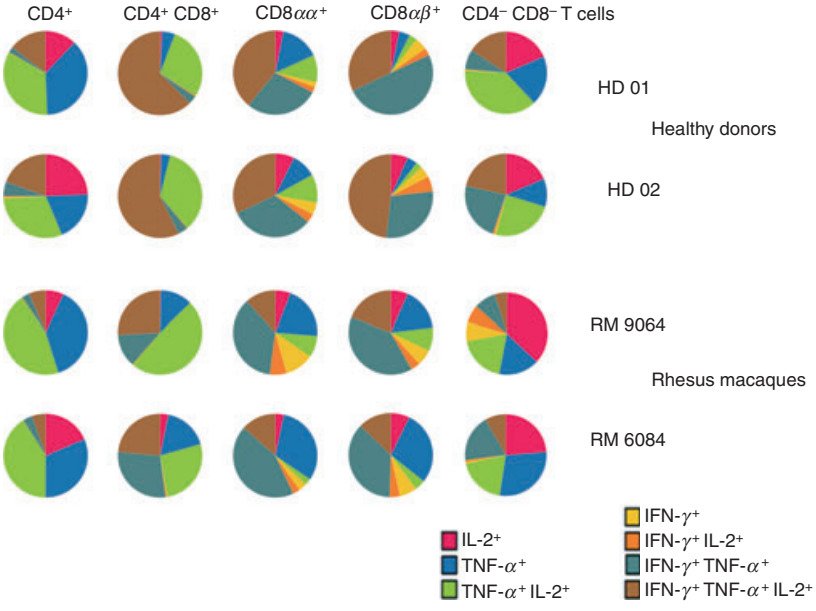

Figure 4. Analysis of polyfunctional T cells. Interleukin-2 (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) production were measured by intracellular cytokine staining on the single-cell level. Two representative individuals from human donors and non-human primates are shown as a paradigm. Note the increased frequency of polyfunctional T-cells in human donors compared with non-human primates.

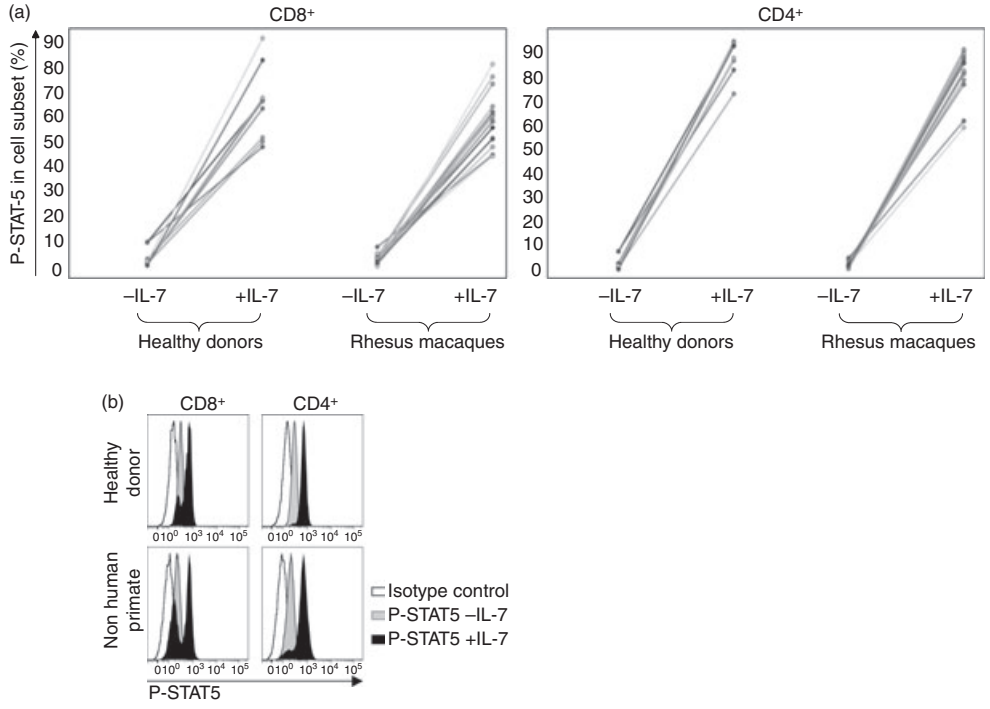

Figure 5. Interleukin-7 (IL-7) -induced signal transducer and activator of transcription 5 (STAT-5) phosphorylation. Phosphorylated (P-) STAT-5 was determined in $CD4^+$ and $CD8^+$ cells before and after exposure to IL-7. (a) Percentage of P-STAT-5 positive cells. Similar levels of constitutive and IL-7-induced STAT-5 phosphorylation in peripheral blood mononuclear cells from human donors and non-human primates. (b) Example of constitutive and IL-7-induced P-STAT-5 in human and non-human primate peripheral blood mononuclear cells determined by flow cytometry.

as measured by STAT-5 phosphorylation in NHPs and HDs. In HDs and NHPs more $CD4^+$ cells than $CD8^+$ cells showed STAT-5 phosphorylation (consistent with higher levels of IL-7Rα expression on $CD4^+$ T cells) but the amplitude (measured by MFI) was not statistically different between $CD4^+$ and $CD8^+$ cells.

Differences in Tregs defined by IL-7Rα expression

The presence of regulatory cells was assessed by expression analysis of CD25 and FoxP3 in the $CD4^+$, $CD8^+$ and $CD4^+$ $CD8^+$ T-cell compartments (gating strategy see Supplementary Fig. S2). In NHPs, the $CD4^+$ T-cell compartment showed the following frequencies: 16·5% (median values) were $CD25^{intermediate}$ ($CD25^{interm.}$) and 0·5% stained for $CD25^{bright}$; in $CD4^+$ $CD8^+$ T cells: 19·6% cells were $CD25^{interm.}$ and 1·4% were $CD25^{bright}$; in $CD8^+$ T cells: 1% were $CD25^{interm.}$ and 0·07% $CD25^{bright}$ (Table 2). Compared with HDs, higher frequencies of $CD4^+$ $CD25^{interm.}$ T cells and $CD4^+$ $CD8^+$ $CD25^{interm./bright}$, and $CD8^+$ $CD25^{bright}$ T cells (21%) were detected in PBMCs from NHPs. Analysis of FoxP3 expression in the different $CD25^{+/-}$ T-cell compartments showed that the majority of $CD25^{bright}$ T cells in NHPs were $FoxP3^+$ (87·5% of $CD4^+$ and 76% of $CD4^+$ $CD8^+$ and $CD8^+$ T cells), whereas only 10–20% of $CD25^{interm.}$ T cells showed FoxP3 expression (Fig. 6a). In contrast, almost no FoxP3 expression could be observed in human $CD4^+$ $CD8^+$ $CD8^+$ T cells that exhibited the $CD25^{interm.}$ phenotype. Analysis of PBMCs from four of eight HDs showed that

Table 2. CD25 expression in $CD4^+$, $CD4^+$ $CD8^+$ and $CD8^+$ T-cell compartments in healthy human donors and non-human primates

	$CD3^+$ $CD4^+$			$CD3^+$ $CD4^+$ $CD8^+$			$CD3^+$ $CD8^+$		
Median	$CD25^-$	$CD25^{interm}$	$CD25^{bright}$	$CD25^-$	$CD25^{interm.}$	$CD25^{bright}$	$CD25^-$	$CD25^{interm}$	$CD25^{bright}$
Rhesus macaques	82**	16·5**	0·5	79·4**	19·6**	1·4*	99	1	0·07*
Healthy donors	95·9	3·4	0·5	96·5	2·3	0·4	98·8	1	0·01

(%) Median values.

**$P < 0·001$, $P < 0·05$ (Mann–Whitney U-test).

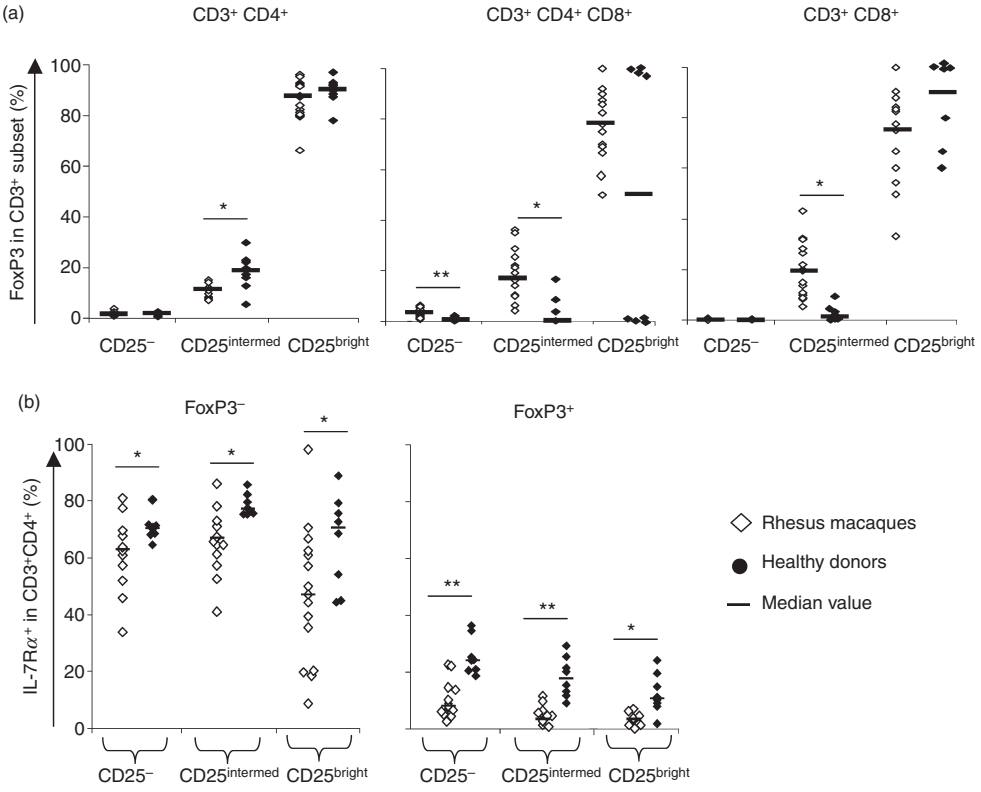

Figure 6. Determination of regulatory T cells based on marker analysis. (a) $CD4^+$ $CD8^-$ (left), $CD4^+$ $CD8^+$ (middle panel) and $CD8^+$ $CD4^-$ (right) T cells were segregated based on CD25 expression and FoxP3 analysis was performed as shown in the supplementary Fig. S2. Note that some $CD8^+$ ($CD4^-$) $CD25^{bright}$ T-cells showed low FoxP3 expression in non-human primates. (b) Interleukin-7 receptor α (IL-7Rα) -expressing T cells in $CD4^+$ $CD8^-$ regulatory T cells (Tregs) based on FoxP3 analysis. Higher percentage of IL-7Rα$^+$ T cells in $Foxp3^-$ $CD25^{bright}$ Tregs in peripheral blood mononuclear cells from humans than in those from non-human primates. $^{**}P < 0{\cdot}001$, $^{*}P < 0{\cdot}05$ (Mann–Whitney *U*-test).

$CD4^+$ $CD8^+$ and $CD8^+$ T cells, which displayed a $CD25^{bright}$ phenotype, stained also positive for FoxP3. In summary, statistically higher frequencies ($P \leq 0{\cdot}05$) of $CD4^{+/-}$ $CD25^{interm.}$ $FoxP3^{+/-}$, $CD4^{\pm}$ $CD8^{\pm}$ $CD25^{interm./high}$ $FoxP3^{+/-}$ and $CD8^{\pm}$ $CD25^{interm./high}$ $FoxP3^{+/-}$ were detected in NHPs than in HDs.

Expression of the IL-7Rα on NHP $CD25^{bright}$ T cells was inversely correlated with expression of FoxP3, which is similar to the situation described in humans.[25] More than 90% of NHP $CD4^+$ $CD8^+$ $CD25^{interm./bright}$ $FoxP3^+$ T-cell subsets did not express the IL-7Rα, whereas the majority of $CD4^+$ $CD8^+$ $CD25^{+/-}$ $FoxP3^-$ (33–67%) were positive for IL-7Rα (% of IL-7Rα expression is shown for $CD4^+$ T cells in Fig. 6b). The same trend was observed in HDs. However, 9% of human $CD4^+$ $CD25^{bright}$ $FoxP3^+$ T cells expressed the IL-7Rα (Fig. 6b), this was not true for the same T-cell subset in NHPs (3·8%). The MFI of IL-7Rα in the percentage of IL-7Rα expressing $FoxP3^+$ cells was decreased compared with $FoxP3^-$ T cells, which indicates a lower number of IL-7Rα molecules/cell compared with $FoxP3^-$ T cells (data not shown).

Discussion

Limitations of any immune composition analysis may be related to the origin of the animals, previous exposure to environmental pathogens and age.[26–29] It has been shown that gender may affect lymphocyte frequencies. In humans, females were found to show higher $CD4^+$ T-cell frequencies,[30] whereas others showed similar[20] or different[31] $CD4^{+/-}$ and $CD8^{+/-}$ T-cell compositions in PBMCs from female and male Chinese rhesus monkeys. The presence of steroid receptors on immune cells[32] may account for differences in lymphocytes in females compared with males. A variety of other factors also impacts on PBMC composition. An increase of peripheral blood lymphocytes can be induced by exercise or stress with preferential mobilization of certain lymphocyte subsets during exercice.[33] The aim of this study was to characterize the immune compartment, compare the phenotype of different T-cell subsets in a large cohort (27 animals) of young female Chinese rhesus macaques using reagents cross-reacting with human and NHP CD markers. The results were compared with a cohort of younger to older

(19–66 years) female and male HDs with the limitations discussed above.

The NHP cohort, like in the human population, is outbred and individual variation is to be expected; to match exactly the age of the HDs and NHPs is not easily feasible because of the limited access to blood from appropriately age-matched individuals. Yet despite gender and age differences between HDs and NHPs, our study provides useful insights into some of the commonalities and differences between human and NHP immune cell compartments.

Differences in T-cell differentiation/homing markers

In this report we describe the composition of different T-cell compartments based on the simultaneous detection of CD45RA, CCR7, CD28 and CD27 in humans, and for the first time in rhesus monkeys. In NHPs and HDs CD8$\alpha\beta^+$ T cells showed a preferential distribution within the precursor CD45RA$^+$ CCR7$^+$ compartment. In PBMCs from HDs, precursor T cells predominantly co-expressed CD28 and CD27 as shown previously by Romero *et al.*[34] We identified CD45RA$^+$ CCR7$^+$ T cells that expressed only CD28 or CD27 and this was not described in their report. Human CD4$^+$ T cells exhibited precursor phenotype and co-expressed CD28 and CD27 as described by Okada et al.[35] Differentiated effector (CD45RA$^+$ CCR7$^-$) T cells represented 19% of the CD4$^+$ T-cell compartment. This frequency is higher than reported by previous studies;[35,36] differences in age,[37] sex, or ethnic composition of the human cohorts may account for these differences. The majority of the CD8$\alpha\beta^+$ and CD4$^+$ T cells (> 70%) in PBMCs from rhesus monkeys co-expressed CD45RA and CCR7. However, the expression of CD28 and CD27 differed from that in HDs: fewer T cells co-expressed CD28 and CD27, most T cells expressed only CD28 (e.g. 40% of CD8$\alpha\beta^+$ T cells). Pitcher *et al.*[3] have characterized T-cell subsets in rhesus macaques based on the expression of markers such as CD45RA, CD95 and CD28, and concluded that CD95 and CD28 expression represented valid markers for the definition of precursor/effector/memory T-cell subsets, whereas the use of CD45RA and CD62L expression failed to identify precursor T cells. CD45RA expression was found on putative memory T cells and cytomegalovirus antigen-experienced cells. In humans, central memory T cells display a CD45RA$^+$ CCR7$^-$ phenotype, and antigen-specific T cells have been found in different T-cell memory compartments.[38] Furthermore, in the report by Pitcher *et al.* the marker CCR7 was not used so it does not exclude the use of CD45RA in combination with other markers (including CCR7) to delineate T-cell subsets.[39,40] Our results show that more CD45RA$^+$ CCR7$^+$ CD28$^+$ CD27$^+$ cells (putative precursor cells) were present in the CD4$^+$ than in the CD8$\alpha\beta^+$ T-cell compartment in NHPs. This observation is consistent with the report by Pitcher *et al.* that the frequency of memory cells increases faster in CD8$\alpha\beta^+$ T cells than in CD4$^+$ T cells. Furthermore, CD45RA$^+$ CCR7$^+$ CD28$^+$ CD27$^+$ CD4$^+$ and CD8$\alpha\beta^+$ T cells were enriched for IL7-Rα^+ T cells (77·4% and 55%, respectively are IL-7Rα^+), suggesting that these cells may indeed represent precursor T cells.[18] The biology of CD45RA$^+$ CCR7$^+$ CD28$^+$ CD27$^-$ T cells in NHPs remains to be defined, they could represent T cells that entered differentiation. Alternatively, they could represent antigen-experienced T cells that regained CD45RA$^+$ CCR7$^+$ expression.[41] A different area in NHP research attempts to reveal why natural simian immunodeficiency virus (SIV)-infection of African NHPs does not lead to disease.[42] A key difference is that NHPs may develop an anti-inflammatory response that prevents chronic activation, and T-cell proliferation.[43,44] Our observation that lower frequencies in NHPs of cytokine-producing cells in CD4$^+$ CD8$^+$, CD4$^-$ CD8$^-$ and CD8$\alpha\beta^+$ T cells after PMA/ionomycin stimulation may indicate intrinsic differences in the levels of activation and T-cell responses between humans and NHPs. Lower levels on T cells of IL-7Rα expression were observed in NHPs, T-cell homeostasis in NHPs may have a lower requirement for IL-7. Interestingly, it was recently described that higher levels of plasmatic soluble IL-7Rα are detected in rhesus monkeys than in humans,[45] suggesting that IL-7Rα shedding could also explain the lower detection of cell surface IL-7Rα in NHPs.

Differences in CD8$\alpha\alpha^+$ T cells

CD3$^+$ T cells that express the CD8$\alpha\alpha$ homodimer have been described in mice[46] and man.[47,48] The CD8$\alpha\alpha$ homodimer was transiently expressed in antigen lymphocytic choriomeningitis virus (LCMV) specific T cells along with markers for increased T-cell survival, i.e. IL-7Rα and Bcl-2.[46] Mice defective in expressing CD8$\alpha\alpha$ homodimers ($E8_I^{-/-}$) showed impaired CD8$^+$ T-cell memory formation. However, other reports could not identify impaired CD8$^+$ T-cell memory in $E8_I^{-/-}$ mice in response to LCMV[49] and influenza virus A[50] (yet in the latter report a correlation between CD8$\alpha\alpha$ expression and elevated levels of IL-7Rα and Bcl-2 was observed). The CD8$\alpha\alpha$ homodimer, a ligand for the non-classical major histocompatibility complex (MHC) molecule thymic leukaemia antigen,[51] is transiently expressed on CD8$\alpha\beta^{+50}$ T cells that down-regulated the CD8β chain. Studies performed on human blood samples identified CD8$\alpha\alpha^+$ T cells as a particular memory T-cell subset[47,48] which is stable over time[52] and enriched in antigen-specific T cells. Our data showed that CD8$\alpha\alpha^+$ T cells are not only present in NHPs, but are also present at higher frequency, in the peripheral circulation of NHPs, and that in HDs and NHPs CD8$\alpha\alpha^+$ T cells were enriched in differentiated T cells compared with CD8$\alpha\beta^+$ T cells.

The NHP CD8$\alpha\alpha^+$ T cells may therefore also represent a memory T-cell subsets for long-lived antigen-specific immune responses:[53] we have previously shown that NHP CD8$\alpha\alpha^+$ T cells, and not CD8$\alpha\beta^+$ T cells specifically proliferate in response to molecularly defined *Mycobacterium tuberculosis* antigens.[53] Down-regulation of the CD8β chain may represent a mechanism that lowers the avidity of the TCR to its MHC–peptide ligand to secure long-term immune cell memory limiting T-cell activation[54] and the risk of activation-induced apoptosis.[55,56.]

Identification of CD4$^+$ CD8$^+$ T cells

Two additional T-cell compartments were present in HDs and at a higher frequency in NHPs: CD4$^+$ CD8$\alpha\alpha^+$ and CD4$^+$ CD8$\alpha\beta^+$ T cells as reported previously.[57–59] Their frequency appeared to be higher in female rhesus monkeys.[20] CD4$^+$ CD8$^+$ T cells stained positive for the degranulation marker CD107a. In contrast to a previous report,[59] CD4$^+$ CD8$\alpha\alpha^+$ and CD4$^+$ CD8$\alpha\beta^+$ T cells in NHPs showed similar frequencies and their maturation/differentiation marker profile reflected the phenotype of the 'conventional' CD4$^+$ CD8$^-$ T cells. We postulate that CD4$^+$ CD8$^+$ T cells represent a specialized compartment of CD4$^+$ T cells formed during the different stages of T-cell differentiation, characterized by CD8 expression. Because the CD4$^+$ CD8$^+$ T cells were endowed with effector capacity (CD107a expression) (model Fig. 7); it could be that CD4$^+$ CD8$^-$ T cells represent a CD4$^+$ T-cell compartment capable of lysing target cells, the co-expression of CD8 enables intracellular calcium levels to be increased, enhances cytotoxicity and may prevent apoptosis[60] upon binding to MHC class I molecules.

IL-17 production in CD4$^+$ CD8$^+$ and CD8$\alpha\alpha^+$ T cells

To examine the role of CD4$^+$ CD8$^+$ T cells, we evaluated IL-17 production in PBMCs from HDs and NHPs in the presence IL-23 and IL-1β.[61] Only data from HDs could be analysed because of the low number of IL-17-positive events in NHP PBMCs. CD4$^+$ CD8$^+$ T cells showed a higher, and CD8$\alpha\alpha^+$ T cells a comparable, frequency of IL-17 production, yet a different profile (more polyfunctional IL-17$^+$ TNF-α^+ IFN-γ^+) as compared with CD4$^+$ (CD8$^-$) T cells. These data support the notion that CD4$^+$ CD8$^+$ T cells appear to represent a distinct CD4$^+$ T-cell memory compartment, in part characterized by IL-17 production. Our results also showed that the differentiation of IL-17$^+$ producing CD8$^+$ T cells occurred preferentially within the CD8$\alpha\alpha^+$ T-cell compartment. Interleukin-17 production by memory CD8$^+$ T cells, displaying a CD27$^+$ CD28$^{+/-}$ CD45RA$^-$ phenotype in humans, was described by Kondo *et al.*[62]

Differences in T cells with putative regulatory or suppressive functions

CD4$^+$ Tregs are characterized by co-expression of FoxP3 and high levels of CD25.[63] We observed comparable frequencies of CD4$^+$ (CD25^{high} FoxP3$^+$) Tregs in PBMCs from HD and NHPs. CD8$^+$ Tregs (CD8$^+$ CD25$^+$ FoxP3$^+$) have been described in humans,[64,65] and in rhesus monkeys.[66] We show that CD8$^+$ Tregs (CD8$^+$ CD25$^{interm./high}$ FoxP3$^+$) were present in PBMCs from NHPs in higher frequencies compared with HDs. The same was true for other T-cell subsets co-expressing FoxP3 and CD25 with putative regulatory functions, i.e. CD4$^+$ CD25^{interm} FoxP3$^+$, CD4$^+$ CD8$^+$ CD25$^{interm./high}$ FoxP3$^+$. The FoxP3 and CD25 can be induced upon T-cell activation, it is exclusively expressed by Tregs.

The observation that NHPs showed a decreased number of *bona fide* IL-7Rα^+ in CD4$^+$ Tregs underlines the fact that differential suppressive functions may be present in NHPs compared with HDs. FoxP3 interacts with the IL-7Rα promoter and facilitates the down-regulation of IL-7Rα in CD4$^+$ CD25^{bright} Tregs;[67] negative staining for

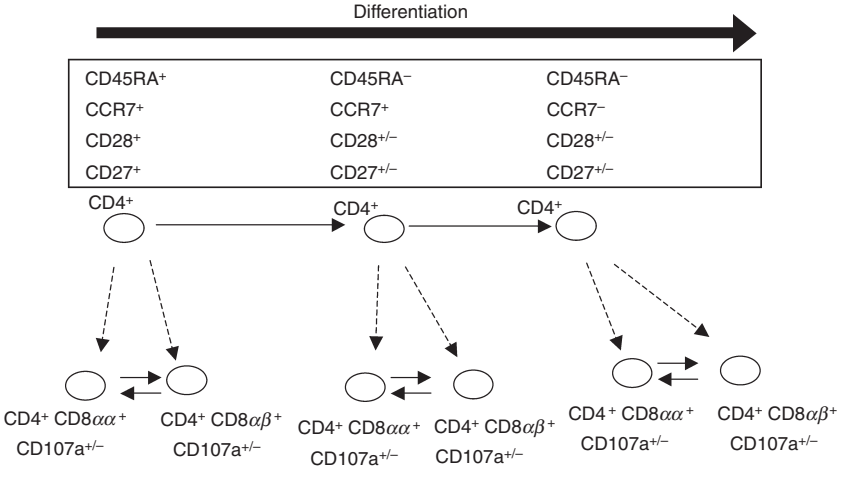

Figure 7. Model of CD3$^+$ CD4$^+$ CD8$^+$ T-cell differentiation. Human donor and non-human primate CD3$^+$ CD4$^+$ CD8$\alpha\alpha^+$ and CD3$^+$ CD4$^+$ CD8$\alpha\beta^+$ T cells expressed CD107a and displayed a very similar phenotype to CD3$^+$ CD4$^+$, suggesting that CD3$^+$ CD4$^+$ CD8$^+$ arise from CD3$^+$ CD4$^+$ and represent a 'back-up' compartment endowed with cytotoxic functions. CD4$^+$ CD8$\alpha\alpha^+$ may arise from CD4$^+$ CD8$\alpha\beta^+$ (or vice versa).

IL-7Rα was postulated as a marker for human Tregs in concert with CD4, CD25 and FoxP3 analysis.[68,69] A low percentage of human Tregs express IL-7Rα and these cells are important in diseases: a recent study showed that human $CD3^+$ $CD4^+$ $CD25^+$ Tregs, which stain positive for IL-7Rα, exhibit an aberrant functional capacity in patients with autoimmune diseases: they exhibit increased proliferation and more IFN-γ/IL-2 production compared with the same cells from healthy individuals.[70] The number of IL-$7R\alpha^+$ expressing $CD4^+$ Tregs was lower in NHPs than in HDs and this may also provide the cellular basis for differential suppressive networks in NHPs.

In summary, we showed, using high content flow cytometry, that the cellular immune system in humans and NHPs exhibited high level of communalities, including a unique $CD4^+$ $CD8\alpha\alpha/\alpha\beta^+$ T-cell population with cytotoxic potential. Differences between humans and NHPs reside in immune cell subsets with long-term memory, i.e. in $CD8\alpha\alpha^+$ T cells and in cells with regulatory functions. This may be biologically important in chronic disease models where inflammatory patterns contribute to immune pathology.

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Disclosures

The authors declare that there is no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Identification of IL-17 producing cells.

Figure S2. Gating strategy to identify Tregs.

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