Developmental induction of human T-cell responses against Candida albicans and Aspergillus fumigatus

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The origin of human T-cell responses against fungal pathogens early in life is not clearly understood. Here, we show that antifungal T-cell responses are vigorously initiated within the first years of life against lysates and peptides of Candida albicans or Aspergillus fumigatus, presented by autologous monocytes. The neonatal responding T-cell pool consists of 20 different TCR-Vβ families, whereas infant and adult pools display dramatically less variability. Although we demonstrate no bias for anti-fungal IL-4 expression early in life, there was a strong bias for anti-fungal IL-17 production. Of note, only T-cells from neonates and infants show an immediate co-expression of multiple cytokines. In addition, only their T-cells co-express simultaneously transcription factors T-bet and RORγt in response to fungi and subsequently their target genes IL-17 and IFNγ. Thus, T-cells of neonates and infants are predetermined to respond quickly with high plasticity to fungal pathogens, which might give an excellent opportunity for therapeutic interventions.

Humans are exposed to fungi such as Aspergillus fumigatus (A. fumigatus) and Candida albicans (C. albicans) e.g. by inhaling spores or by ingesting contaminated food. Especially fungi such as C. albicans have co-evolved with humans and even colonize their body surfaces. Colonization by Candida yeast is thought to occur at an early age, with the organism being acquired during passage through the birth canal, nursing, or from food. An opportunistic infection by C. albicans is initiated rarely, but in particularly frequent from 4 weeks to 9 months of age. Most individuals affected from fungal pathologies are immunosuppressed, patients with disrupted mucosal barriers, patients taking multiple antibiotics, and young infants. Surprisingly, overall mortality of pediatric candidemia is high and did not improve over the past decade1.

Likely, fungi-exposure induces long lasting adaptive immune responses2,4 and especially the CD4 T cell compartment of the adaptive immune response is critically involved in efficient fungal defence, as demonstrated in HIV patients having low CD4 T cell counts5. Naïve CD4+ T cells differentiate when encountering their antigen presented by APC, into different T-helper (Th) subsets, i.e. Th1, Th2, Th9, Th17, Th22, which have signatory cytokine expression6. Th1 cells produce the cytokines IL-2, IFNγ, and TNFα and are decisive for host defence against intracellular pathogens7,8. For Th2 cells, responses are associated with the secretion of cytokines such as IL-4, IL-5, IL-10, IL-13, and IL-24. In response to IL-1β, IL-6, and TGFβ2,10 Th17 cells are differentiated and maintained that produce IL-17. Furthermore, peripheral Th cells such as Th17 cells show to some extend flexibility meaning that they gain characteristics of other lineages e.g. Th17 cells are able to become Th1-like cells11,12. T cell responses to C. albicans have been described as a finely tuned balance between Th1, Th17 and Treg subsets13. The clearance of infections caused by C. albicans on mucosal surfaces was shown to be driven by Th17 responses14, indeed, whereas Th1 and Th17 cells are regarded to be the cell types in providing immune response to oral and dermal candidiasis2,15. In regard to A. fumigatus, specific effector/memory CD4 T cells derived from peripheral blood of healthy adult donors displayed a Th1 phenotype16 and A. fumigatus-expanded Th1 cells are currently being tested for adoptive therapy upon stem cell transplantation17 (Trail EudraCT 2013-002914-11). However, the antifungal Th17 responses of infected patients are more modest18 and appear to require further development.
lung-derived *Asperillus*-specific T cells of the effector/memory subpopulation show a Th17 phenotype with notably production of IL-17, implicating the involvement of a complex adaptive immune response in clearance of fungal pathogens.

Besides recognizing that the immune system in the neonatal and infant period is distinct from the adult one, not much is known about its abilities to defend pathogens antigen-specifically. At birth, being suddenly exposed to antigens and pathogens of the environment, an immediate defence is required while inflammation is kept low in order to protect developing tissues. Recently, a general characteristic production of Th2 cytokines by human neonatal T cells has been reported, whereas, Th1 responses were reported to be weaker. In addition, neonatal T cells also produce high frequencies of CXCL8 upon stimulation. In addition, especially IL-17 is enhanced produced in term and preterm infants compared to adults. Supporting the importance of IL-17, mutations in the IL17RA causes chronic mucocutaneous candidiasis starting at 6 months of age. Although rare, data on antigen-specific responses on cord blood T cells is arising using fungal-stimulation combined with polyclonal expansion procedures, which gives evidence that naive T cells at birth are able to respond to *C. albicans*. Taken together, knowledge on initiation of specific, antifungal T cell responses of neonates, infants, and even children are limited and, thus, has to be complemented if we are to learn how early-life adaptive immune responses affect paediatric and in the long run - adult health.

**Materials and Methods**

**Samples.** PBMCs were obtained from leukocyte reduction filters (Sepacell RZ-2000; Asahi Kasaei Medical) supplied by the Institute of Transfusions Medicine and Immunohaematology at the University Hospital of Magdeburg. Cord blood samples were obtained from umbilical cord veins immediately after birth from the Women's Clinic of the University Hospital of Magdeburg. The blood and adenoids were obtained from children (age 9.5–12 years of age) suffering from adenoid hypertrophy through surgical excision and supplied by the Department of Otolaryngology of the Medical University Hospital in Magdeburg. All infants, children, and adults had no history of recurrent infections or inflammations and within the last 4 weeks no cold/flu or intake of antibiotics. At the time of surgery or donation, donors were clinically free of infection. The study had formerly been approved by the Clinical Research Ethics Board of the University of Magdeburg (certificates 06/11, 79/07 and 26/12), and all donors and parents provided written informed consent in accordance with the declaration of Helsinki.

**Cell purification and cell culture.** Mononuclear cells were obtained from cord blood (CB), peripheral blood (PB) of healthy donors, and of surgically excised adenoids of infants suffering from non-inflammatous heat-inactivated (h.i.) *C. albicans* (10 µg/ml, ATCC 10231) (Fig. S1), h.i. *A. fumigatus* (10 µg/ml, ATCC MYA-4609; protocol of Gaudar et al. ), Tetanus Toxoid (TT, 10 µg/ml) from *Clostridium tetani* (Calbiochem), staphylococal enterotoxin B (SEB, 1 µg/ml) from *Staphylococcus aureus* (Sigma Aldrich), or fungal peptides PepMix Candida (MP65, 1 µg/ml) (JPT Peptides Technologies GmbH) over night at 37 °C in RPMI 1640 medium (Biochrom). The RPMI 1640 medium was supplemented with 10% Fetal Bovine Serum (Gibco/Life Technologies GmbH); 10 µg/ml streptomycin; and 100 µ/ml penicillin (Life Technologies GmbH). Monocytes were washed twice prior co-culturing with T cells.

CD4+CD45RA+ T cells or recent thymic emigrants (CD4+CD45RA-CD31+) were enriched to high purity (>98.5%) by magnetic beads separation using autoMACS-Pro using human naive CD4+ T Cell Isolation Kit or human CD4+ Recent Thymic Emigrant Isolation Kit (Miltenyi Biotec), respectively (Fig. S2). Only samples of >99.4% CCR7+ (Figs 1–5) or >CD31+ T cells (Figs 6 and 58) of CD4+CD45RA+ T cells were considered naive and used for cell assays. In 96-well plates, 5 × 10^6/ml purified T cells were stimulated with the fungi-pulsed CD14+CD16- non-classical monocytes (2.5 × 10^5/ml purified monocytes) at a ratio 2:1 (T-cells/monocyte) for 3 or 6 days. For blockade of HLA-DR, monocytes were incubated with neutralizing anti-HLA-DR mAb (10 µg/ml, L249, purified from hybridoma, controlled by Western blotting and competitive FACS analysis), for 30 min at 37 °C in RPMI 1640 medium (Biochrom, supplemented as described above) prior to their maturation with antigens. Matured monocytes were washed twice, again incubated with anti-HLA-DR mAb for 30 min at 37 °C and co-cultured with T-cells as described above. Viability of monocytes upon anti-HLA-DR mAb treatment was controlled by manual gating of CD14+CD16-/Annexin V~/propidium iodide~ cells (Data not shown).

Cytokines IL-6 and IL-1β were neutralized using anti-IL-6 (10 µg/ml, MQ2-13A5, Biolegend) and anti-IL-1β (10 µg/ml, 8516, R&D Systems) antibodies. For TLR inhibitor experiments, MyD88 inhibitor Pepinh-MYD (50 µM, InvivoGen) and anti-TLR2 (10 µg/ml, B4H2, InvivoGen) were used. For positive control, T cells were stimulated routinely with microbeads coated with anti-CD3 (1 µg/ml, UCHT1) and anti-CD28 (2 µg/ml, CD28.2) (both Biolegend) at a cell to bead ratio of 2:1.

**Flow cytometric analysis.** Naïve (CD4+CD45RA-CD45RO-CCR7+CD25-CD8- T cells were identified using specific fluorescent labelled antibodies (anti-CD4 (RPA-T4), anti-CD45RA (HI100), coupled anti-CD45RO (UCHL1), anti-CD25 (BL96); anti-CD8 (RPT-T8), and anti-CCR7 (GO43H7) (all Biolegend)) and isolated using FACs Aria (BD Bioscience) from the enriched CD4+CD45RA+ T cells. FcR Blocking Reagent (Miltenyi Biotec) was used to block Fc receptors according to the manufacturer's instructions.

To determine cell proliferation, T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes/ Life Technologies GmbH) according to the standard protocol. CFSE-dilution at different time points was then analysed by flow cytometry.

Prior to intracellular cytokine analysis, naïve (CD4+CD45RA-CD8-CD25-) or recent thymic emigrant (CD4+CD45RA+CD8-CD25-CD31+) T cells were pulsed with 10 ng/ml PMA, 1 µg/ml Ionomycin, and 5 µg/ml Brefeldin A (Sigma-Aldrich) for 4h. Cells were fixed with 2% paraformaldehyde (Morphisto GmbH) in PBS for
20 min, following permeabilized with 0.5% saponin (Sigma-Aldrich) in PBS/BSA, and then incubated with the following antibodies: anti-IL-17A, anti-IFNγ, anti-CD4, anti-CD3 (all Biolegend, San Diego, USA). To analyse activation-induced surface molecules by flow cytometry, cells were harvested and stained with anti-CD4 (RPA-T4), anti-CD3 (SK7); anti-CD45 (HI30); anti-IL-2 (MQ1-17H12); anti-IL-4 (MP4-25D2, for detection of mature IL-4); anti-IL-4 (8D4-8; for detection of IL-4 isoform) and anti-TNFα (Mab11) (all Biolegend, San Diego, USA).
To measure transcription factors by flow cytometry, cells were fixed with 4% formaldehyde (Carl Roth) in PBS for 10 min at 37 °C followed by permeabilization in ice cold 90% methanol (Carl Roth) for 30 min, and then stained with antibodies for specific transcription factors anti-RORγt (eBioscience) and anti-T-bet (Biolegend).

For the assessment of TCR-Vß expression we used IOTest Beta Mark TCR-Vß Repertoire kit (Beckman Coulter) for quantitative analysis of the most abundant 24 TCR-Vß clonotypes. To identify activated T cells, cells were co-stained with anti-CD4 (RPA-T4), anti-CD3 (SK7); anti-CD45 (HI30), anti-CD69 (FN50), and anti-CD25 (M-A251) (all Biolegend). All flow cytometric analyses were performed using FACS Canto II (BD Biosciences) together with FACSDiva software (BD Biosciences) to collect and compensate the data and FlowJo software (FlowJo LLC) for final data analysis.

**EliSpot assays.** ELISPOT plates (Millipore 96-well MultiScreen HA; Millipore) were coated with 2 mg/ml anti-human IL-17–specific mAb (eBio64CAP17; eBioscience) in PBS. Cells were cultured in the presence of PMA and ionomycin for 18 h at 37 °C/5% CO2 and plated at a density 1,25 × 10⁵ cells/well in the ELISPOT plate.
Cytokines IL-17 and IFNγ were detected with biotinylated anti-human IL-17 or IFNγ Abs, respectively (eBio-64DEC17; eBioscience) and developed using 0.3 mg/ml extravidin-alkaline phosphatase (Sigma-Aldrich) and an Alkaline Phosphatase-substrate Kit III Vector Blue (Vector Laboratories). Spots were counted with ImageJ.

**Analysis of cytokines in T cell culture supernatant.** Levels of IFNγ, TNFα, IL-2, and IL-17A, in human T cell assay supernatants were measured using LEGENDplex human Th Cytokine Panel (Biolegend) according to the manufacturer’s instructions. Samples were diluted 1:3 in assay buffer. Analyses were performed using FACS Canto (Becton Dickinson) and LEGENDplex analysis software (VigeneTech Inc.).

**Statistical analyses.** Statistical analyses and cumulative data presentation were performed with Prism 7 (GraphPad 7 software Inc.). We used Shapiro-Wilk test for testing of normality. Comparison of distribution was performed using two-tailed Student’s t-test or Wilcoxon test for comparison of two parameters; one-way-ANOVA or Kruskal-Wallis test for multiple comparisons depending on the results of Shapiro-Wilk test, with P < 0.05 (**), P < 0.01 (***), and P < 0.001 (****) indicating statistically significant differences.

**Results**

**T cells of neonates and infants respond to C. albicans and A. fumigatus extensively.** As neonatal and infant T cells are thought to be hyporesponsive, we investigated whether naïve CD4+ T cells from neonates and infants are able to respond efficiently to C. albicans and A. fumigatus antigens presented by autologous APCs (Fig. S1), respectively. Isolation of CD4+CD45RA+ T cells from neonates, infants, children, and adults which had no history of recurrent infections or inflammations revealed routinely >99.4% CD3+ cells (adults >98.6%) and showed similar results as FACS sorted CD4+CD45RA+CCR7+ T cells (data not shown, Fig. S2), and were
**Figure 4.** Fungi specific Th1 cytokine expression by T cells of different age groups. CD4⁺CD45RA⁺ T cells from neonates, infants, children, and adults were stimulated with *C. albicans* (orange) or *A. fumigatus* (blue) (as in Fig. 1) for 3 and 6 days respectively (A) Frequency of T cells expressing intracellular IL-2 (left panel), TNFα (middle panel) or IFNγ (right panel) was determined by flow cytometry. (B) Determination of IL-2 (left panel), TNFα (middle panel) or IFNγ (right panel) cytokine release of CD4⁺CD45RA⁺ T cells of neonates, infants and children or adults by LegendPlex which were either stimulated or not for 3 days. (C,D) CD4⁺CD45RA⁺ T cells were stimulated with *C. albicans* (C) or *A. fumigatus* (D) as in A, and the cells expressing single or multiple cytokines IL-2, TNFα, and IFNγ were determined by flow cytometry and analysed by Boolean gating and shown as fraction of all CD4⁺ T cells in a pie chart. The subsets that simultaneously express no (grey), one (blue), two (yellow) or three (red) different cytokines are grouped by colour. The data are representative of at least 5 donors. Cumulative results are shown and each dot in (A) and (B) represent a different donor. The error bars in figures denote ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, as determined by one-way Anova with Tukey post hoc test (A) or Kruskal Wallis with Dunn's post hoc test (B).
Figure 5. Age-dependent IL-4 production by fungi specific T cells. (A,B) CD4+CD45RA+ T cells from
 endemic, infants, children, and adults were stimulated with C. albicans (orange) or A. fumigatus (blue) (as in
 Fig. 4) for 3 (left panel) and 6 days (right panel) respectively, and analysed for the expression of intracellular
 un-glycosylated IL-4 isoform (upper panel) and mature IL-4 (lower panel). Cumulative results are shown and
each dot represents a different donor. The error bars in figures denote ±SD. *p < 0.05, **p < 0.01, ***p < 0.001,
 ****p < 0.0001, as determined by one-way ANOVA with Tukey post hoc test.
Figure 6. Fungi specific T cells produce IL-17 in an age dependent manner. CD4+CD45RA+CD31+ T cells from neonates, infants, children, and adults were co-cultured with monocytes pulsed with C. albicans- or A. fumigatus-lysates. (A,B) The frequency of T cells expressing signature Th17 molecules IL-17 (A) and RORγt (B) were analysed by flow cytometry at day 3 (upper Panel) and day 6 after stimulation (lower panel) (C) Bar graph representing the ELISPOT analysis of the quantitative IL-17, produced by the T cells from neonates, infants, children, and adults were co-cultured with monocytes pulsed with C. albicans- or A. fumigatus-lysates and RORγt from neonates, infants, children, and adults were co-cultured with monocytes pulsed with C. albicans- or A. fumigatus-lysates. (D) Determination of IL-17A cytokine release of CD4+CD45RA+ T cells of neonates, infants and children or adults by LegendPlex which were either stimulated or not for 3 days as described in (A). (D) Bar graph showing IL-17 expression by CD4+CD45RA+CD31+ T cells of neonates, adults, and infants of 0.5-2 years old, stimulated for 6 days as described in (A) in the presence or absence of neutralizing antibodies for IL-1β,

Resting

IL-6

Resting

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A. fumigatus

Adults

IL-6

IL-6

C. albicans

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IL-1γ

IL-17

IL-17, IL-4

E

F

Neonates

Adults

0.5-2 years

3-5 years

Resting

C. albicans

150

A. fumigatus

150

Resting

A. fumigatus

150

A. fumigatus

150
In addition, to underpin the antigen specific responsiveness towards *C. albicans*, we stimulated CD4+ CD45RA+CD31+ T cells 3 days with monocytes matured with *C. albicans*-antigens or with a commercial *C. albicans* peptide mix consisting of 92 peptides of *C. albicans*, respectively (Fig. 2D). To more clearly visualize the antigen-specificity and the age-dependent effect of *C. albicans*, a synthetically generated peptide mix of *C. albicans* proteins was used. In line with our results using fungal lysates (Fig. 2A) results from *C. albicans* peptide mix show that CD4+CD45RA+CD31+ T cells from neonates are activated much more frequently than from children and adults (Fig. 2D). Both antigenic stimuli unambiguously induced CD25 and CD69 expressing cells in comparison to unstimulated conditions. Although fungus-pulsed APCs were washed prior contact with T cells, T cells were pre-incubated with the inhibitor of TLR signalling molecule MyD88 or blockade of TLR2 with specific antibodies and showed no effect on stimulation (Fig. S4). Activity of TLR inhibitor in these experiments was controlled on SEB-activated monocytes (Fig. S5). These results indicate that the compartment of naive T cells of neonates and infants is ready to mount vastly a response against fungal-antigens presented on autologous APCs and - at least for *C. albicans* - even stronger than stimulated naive T cells of older children or of the adult naive T-cell compartment.

**Pre-existing T cell repertoires of fungus reactive T cells.** To investigate the TCR diversity of the responding cells, we isolated naive T cells to a purity of >99.4% CD31+ of CD4+ CD45RA+ T cells from infant donors of different ages and determined the quantitative and qualitative expression of TCR-Vβ subpopulations against *C. albicans* and *A. fumigatus* (IOTest Beta Mark TCR-Vβ repertoire kit). Within 3-days of fungus-specific stimulation, we found that activated CD25+ CD4+ T cells showed a non-random usage of the TCR-Vβ repertoire (Fig. 3). Interestingly, highest number of different Vβ subfamilies was used by CD25+ CD4+ T cells from neonates (Fig. 3D,E). However, a dramatic reduction in TCR-Vβ usage against fungi was observed between T cells from neonates and those of small infants aged 0.5–2 years. Subsequently, number of Vβ subfamilies used against fungi increased steadily with age from infants to adults. Nevertheless, many different Vβ families were uniquely used for the antifungal T-cell response of neonates. Based upon these results, we examined the possibility that Vβ subfamilies missing in the naive compartment might reappear in the memory compartment of adults. Compared with the naive compartment, the number of Vβ families used in memory pool of adults is significantly increased (Fig. 3D,E). The TCR frequency from donors of variant age groups that use 3 different Vβ families (Vβ9, Vβ16, Vβ17) are plotted as examples in Fig. 3A–C. TCR distribution appears to be varying in resting cord blood T cells with low frequencies of T cells (0.45±0.45%) expressing Vβ16 and a high frequency of them (8.9±1.9%) expressing Vβ9. Intriguingly this pattern of TCR distribution was not observed after T cell stimulation, as antigen-specific stimulation of neonatal T cells with *C. albicans*- or *A. fumigatus*-pulsed monocytes led to an increased usage of Vβ16 and Vβ17 in comparison to unstimulated conditions. The expression pattern of TCR-Vβ subfamilies in infants and children show a predominant usage of Vβ3, Vβ5.2, Vβ13.6 and Vβ18 in reply to fungal-antigens that differs from adults. Of note, activated T-cells expressing Vβ8 and Vβ21.3 were not detectable in response to fungal antigens (Fig. 3F). However, many Vβ families were uniquely used in the antifungal T-cell response of neonates and infants. These data show that the antigen-specific T-cell responses to *C. albicans*- and *A. fumigatus*-pulsed monocytes use a broad repertoire implying that T cells of neonates and young children are predetermined to expand to fight fungal pathogens.

**C. albicans and A. fumigatus mediate Th1 cell differentiation in an age dependent manner.** To decipher the age-dependent diversity of fungi-specific T cells in detail, we first analysed expression and accumulation in the supernatants of Th1-like cytokines, namely IL-2, TNFα and INFγ. CD4+ CD45RA+ T cells from representative donors of different ages in response to *C. albicans*- and *A. fumigatus*-pulsed monocytes are shown in Fig. 4A,B. Fungus-specific production of Th1-associated cytokines IL-2 and INFγ by CD4+ CD45RA+ T cells from neonatal samples compared to T cells of infants or adults after stimulation for 3 and 6 days, whereas all donors examined responded similarly to *A. fumigatus* in inducing TNFα producers (2–3%). No difference could be detected between enriched CD4+ CD45RA+ and CD4+ CD45RA+CD31+ T cells at any age group in frequency of cytokine responders to *C. albicans* or *A. fumigatus* (Fig. S6). To ensure that difference in responding T cells indeed translates into differences of secreted TNFα, it was analysed in supernatants from 4 donors of each of the 5 age-groups (Fig. 4B). Indeed, significantly enhanced concentrations of TNFα were monitored in samples of neonates.

By analysing flow cytometric data with Boolean gating, multifunctional T cells were characterized to determine functionality of the T-cell response following stimulation with *C. albicans-* or *A. fumigatus*-antigen-matured monocytes. Although Th1-like cells were observed to be developed at any age, analysis of multi-functional CD4+ CD45RA+ T cells showed that T cells isolated from neonates, infants, and children up to 5 years of age developed double and triple producers already after 3 days of stimulation with *C. albicans*, while T cells from adults did so only at a later stage (Fig. 4C). In addition, significantly different frequencies of cytokine producers were initiated by each fungus; of note, single producers were mainly induced by both of them in T cells from neonates and children up to 2 years of age (Fig. 4D).
Th-2-like cytokine production is initiated in response to *A. fumigatus*. As infants are discussed to have a bias for Th2-like responses\(^4\), we investigated the frequency of Th2-like cells expressing cytokines IL-4 and IL-13 in response to fungi (Fig. 5, S9). In general, the frequencies of T cells responding against *A. fumigatus* with unglycosylated IL-4 production were 2-4 times higher than those against *C. albicans* across all age groups. Interestingly, a frequency of 5% unglycosylated IL-4 expressing T cells are detectable after 3 days of stimulation in all age groups implying that the IL-4 machinery is indeed activated in response to *A. fumigatus* independently of age (Fig. 5A). Mature IL-4 producers were detectable upon 6 days after beginning of the stimulation with *A. fumigatus* in all age groups, with adults displaying a frequency a mature IL-4 producers as high as glycosylated IL-4 producers after 3 days of stimulation (Fig. 5B). At day 6, *C. albicans*-specific responses also contained IL-4 producers (2%), but with no significant age differences. Of note, IL-13 expression is not significantly induced (Fig. S9). However, taken together, Th2 machinery is switched on in response to fungi at any age.

**Neonates respond to *C. albicans* and *A. fumigatus* with IL-17 producing T cells.** For studying age-dependent peculiarities in IL-17 production, we isolated CD4\(^+\)CD45RA\(^-\) CD31\(^+\) T cells and stimulated them with *C. albicans*- or *A. fumigatus*-pulsed monocytes. The quantitative analysis of *C. albicans*-specific cytokine expressing cells by flow cytometry, soluble cytokine in supernatants and cytokine secretion by EliSpot assay, displayed highest frequencies of IL-17\(^+\) fungus-specific T cells in neonatal samples compared to those from infants or from adults (Fig. 6A,C,D). Also the frequency of cells expressing IL-17 transcription factor ROR\(\gamma\) is 2 times more in T cells from neonates than in T cells from adults (Fig. 6B). Next, co-expression of IL-4 with IL-17 was monitored showing higher frequencies in adults after stimulation with *A. fumigatus*, than from neonates or children up to 5 years of age (Fig. 6E).

To further investigate whether the enhanced induction of IL-17 by neonatal T cells upon stimulation with fungi is dependent on IL-18 and IL-6, we isolated CD4\(^+\)CD45RA\(^-\) CD31\(^+\) naive T cells from donors of different ages as indicated and stimulated them with specific fungi in the presence or absence of blocking antibodies against IL-18 and IL-6. As shown in Fig. 6F, neutralization of IL-18 and IL-6 by specific antibodies significantly reduced the generation of IL-17 producers by both fungi in all age groups.

Next, we investigated the capacity of *C. albicans*- and *A. fumigatus*-primed IL-17 expressing T cells to co-produce IFN\(\gamma\). Upon fungus-specific stimulation, T cells from neonates, infants, and children displayed an ability to co-produce cytokines IL-17 and IFN\(\gamma\) (Fig. 7A), which was hardly detectable in responding naive T cells from adults. Co-producing T cells appeared indeed only in the memory compartment of adults (Fig. 7B). As co-production of these 2 cytokines would require transcription factors ROR\(\gamma\)-t and T-bet to be co-expressed in individual cells, the expression of these transcription factors were deciphered in stimulated CD4\(^+\) CD45RA\(^-\) CD31\(^+\) T cells by FACS analysis. Indeed, a high frequency of T cells from neonates but not adults unambiguously co-expressed ROR\(\gamma\)-t and T-bet (Fig. 7C). These results demonstrate that early in life, a high plasticity of Th subsets exists.

**Discussion**

In this study, we investigated the antigen-specific cellular and molecular mechanisms of differentiation of the naïve CD4\(^+\) T cells from neonates, infants, children, and adults in response to *C. albicans* and *A. fumigatus*. Our data revealed the novel observation that unlike T cells from adults, especially in early life, a broad repertoire of antifungal naïve CD4\(^+\) T cells respond with vast proliferation. Responding T cells showed high frequencies of IL-17 producers and co-expression of transcription factors of different Th lineages. Th cell differentiation against fungi was indeed age-dependent with signatory elements; however, the anti-fungal T cell responses were strongly determined by each individual pathogen.

Within fungus-activated T cells, we found a non-random usage of TCR V\(\alpha\) repertoire. To our knowledge, we are the first to show the TCR V\(\alpha\) repertoire in response to *C. albicans*- and *A. fumigatus*-antigens. As the T cell response is fast and extraordinarily high for neonates, a predetermined repertoire with inherited fungus-specific regions seem to be likely, which also have been recently described for malaria-antigens and a respiratory syncytial virus fusion glycoprotein\(^34,35\). This brings us back to the main function of the immune system: fighting pathogens and it might be likely that even a more predetermined repertoire against pathogens will be identified in the near future. The expression pattern of TCR V\(\beta\) subfamilies in infants and children show a predominant usage of V\(\beta\)3, V\(\beta\)5,2, V\(\beta\)13.6 and V\(\beta\)18 in reply to fungal-antigens which differs a lot from adults. In terms of T cell repertoire of naïve T cells respond with vast proliferation. Responding T cells showed high frequencies of IL-17 producers and co-expression of transcription factors of different Th lineages. Th cell differentiation against fungi was indeed age-dependent with signatory elements; however, the anti-fungal T cell responses were strongly determined by each individual pathogen.

Within fungus-activated T cells, we found a non-random usage of TCR V\(\beta\) repertoire. To our knowledge, we are the first to show the TCR V\(\beta\) repertoire in response to *C. albicans*- and *A. fumigatus*-antigens. As the T cell response is fast and extraordinarily high for neonates, a predetermined repertoire with inherited fungus-specific regions seem to be likely, which also have been recently described for malaria-antigens and a respiratory syncytial virus fusion glycoprotein\(^34,35\). This brings us back to the main function of the immune system: fighting pathogens and it might be likely that even a more predetermined repertoire against pathogens will be identified in the near future. The expression pattern of TCR V\(\beta\) subfamilies in infants and children show a predominant usage of V\(\beta\)3, V\(\beta\)5,2, V\(\beta\)13.6 and V\(\beta\)18 in reply to fungal-antigens which differs a lot from adults. In terms of T cell repertoire of resting CD4\(^+\) CD45RA\(^+\) T cells from donors of different ages, our analysis confirms and extends previous results from total CD4\(^+\) T cells in whole blood\(^56,58\), besides a lower frequency of V\(\beta\)9 chain but a higher frequency of V\(\beta\)9 chain in isolated neonatal naïve T cells compared to total CD4\(^+\) T cells\(^56\). However, the relative TCR V\(\beta\) repertoire of resting T cells does not reflect the T cell repertoire pattern upon fungus-specific activation. It is known from literature that some of the most significant TCR clusters in different human donors seem to be associated with common viral pathogens such as parvovirus b19, influenza, CMV and Epstein Barr virus\(^48\). Beyond that, *Segmented Filamentous Bacteria*, members of the gut microbiota of rodents, fish and chickens, induces in mice a strong Th17 response characterized by an enrichment of V\(\beta\)14 TCR within the Th17 cells. These V\(\beta\)14 TCR Th17 cells do not respond to *Listeria monocytogenes*, a strong inducer of Th1 cells\(^60\). In addition, it was also shown that a naïve T cell population expressing a fixed TCR repertoire occurs in response to commensal bacteria in colonic regulatory T cell population but not in effector T cell pool\(^11-14\). Together with these previous studies, our findings strongly suggest that a particular repertoire exists for certain antigen specificity.

In case of fungi provocation, Th17 cells especially play an important role in defending the organism\(^44\). Our data show that CD4\(^+\) CD45RA\(^-\) CD31\(^+\) T cells from neonates display a particularly high signatory IL-17 response to *C. albicans* in a fast manner in comparison to *A. fumigatus*. A signatory IL-17 production by T cells from neonates upon unspecific, polyclonal stimulation has also been reported\(^45\). Therefore, it is likely that the quick IL-17 response especially against *C. albicans* at birth is predetermined by a pronounced usage of TCR V\(\alpha\).
terms of co-evolution of Candida A. and the human immune system, it is likely that neonates are confronted with Candida – at least – during birth through vertical transmission from the mother’s microbiome, thus, it is life-saving for neonates to be able to initiate anti-Candida reactions quickly. Thereafter in life, IL-17 response is decreased against C. albicans and almost absent against A. fumigatus. Recently, in samples from adult donors an expansion of Treg cells rather than naïve T cells in response to A. fumigatus was described. Aspergillus-specific T cells from PBMCs of adult healthy individuals were shown to have a predominant Th1-like phenotype, whereas C. albicans-reactive T cells have been shown to produce mainly IL-17. According to our data, these cells likely are reactivated from the effector/memory pool, as in our hands naïve adult T cells were not able to produce substantial amounts of IL-17. Indeed, we could detect some of these cells within the fungi-specific stimulated memory/effector pool (Fig. 7B). High amounts of Aspergillus-specific IL-17-expressing T cells so far could only be observed in lung of adult COPD patients. We have also observed Aspergillus-specific IL-17-expressing T cells from adults in our hands, but these cells also displayed a strong Th2-like phenotype at the same time. Thus, it would be interesting to see whether the IL-17 expressing cells from COPD patients indeed co-express IL-4, especially in response to A. fumigatus as it was reported for patients with severe asthma and this beneficial information could open doors to design future therapies.

Our results demonstrate that fungus-matured monocytes from neonates as well as from children and adults are potent APCs. Monocytes show potent cytokine production and CD14 and CD16-coexpression in response to fungal antigens indicating that monocyte responses to fungi at birth and early in life are not deficient. Whereas we cannot detect significant difference of fungus-matured monocytes between ages (Fig. S1B),
others reporting contrasting results. For example, a lower capacity of umbilical cord blood monocytes to produce IL-13 and TNFα in response to Tuberculoid Purified Protein Derivative (PDD) was reported to be due to a functional immaturity of cord blood monocytes at the cellular level. Seemingly at odds with these results, previous reports have also demonstrated that monocytes from cord blood express similar or higher levels of TNFα, IL-6 and IL-1α in response to peptidoglycan or TLR agonist panel. At first sight discrepancies could be due that suboptimal or artificial provocations of the monocytes were monitored and could indeed reveal age-related differences. Similar maturation of monocytes in our setting might be due to applying physiological proteins of pathogens in excess, which likely equals out age-related differences. Appealing from an evolutionary point of view, this would ensure that monocytes at any age are able to become potent APCs in response to fungal threat.

In line with the previous findings that IL-6 and IL-16 produced by APCs, are important for inducing IFNβ production by CD4+ T cells, we have observed that blockade of IL-6 or IL-18 or both reduced IL-17 producers in fungus-specific CD4+CD45RA+CD31+ T cells from all age groups. The importance of IL-1β secretion during C. albicans infection is highlighted by the findings that mice deficient in IL-1β receptor are highly susceptible to disseminated candidiasis. However, an approximately 50% total Th17 producers were IL-6 and IL-18 independent, indicating that the residual Th17 cells could be originated intrathymically (nTh17 cells). Alternatively IL-23 might also take over the differentiation of Th17 cells. Stimulation of CD4+CD45RA+CD31+ T cells with fungi-pulsed monocytes also displayed a co-expression of cytokines IFNβ and IL-17 simultaneously, with higher frequencies of these cells detected in neonates and infants. Others have shown that Th1/Th17 cells were also detected at low levels in C. albicans stimulated adult PBMCs, however our data implicate that these cells likely arise from the memory pool (Fig. 7A). In accordance with the cytokine production profile, the transcription factor analysis also clearly demonstrated that CD4+CD45RA+CD31+ T cells from neonates expressed high levels of ROHit and Th1/Th17 simultaneously in response to fungal-antigens; these differences in Th1/Th17 subpopulation likely points towards age-related differences in plasticity of T cell differentiation. These results are in line with studies showing that naive CD4+ T cells generate precursors that are still multipotent and that they are able to produce a heterogeneous progeny, as well as plasticity of T cells in the human immune response. More precise knowledge of this plasticity of Th cells from neonates and infants could open new avenues for therapy aiming for re-programming the most optimal response.

Taken together, our study provides a better understanding of age-related immune responses against C. albicans and A. fumigatus, two harmful human pathogens. Here, we show that subpopulations of CD4+ T cells especially from neonates and infants respond extensively from birth on to C. albicans and A. fumigatus without a bias for Th2. Although cytokines were produced at any age, T cells of neonates show a signatory IL-17 response to fungi accompanied by IL-17/IFNγ co-expressing T cells. Together with rapid generation of multifunctional anti-fungal T cells, the adaptive immune system of neonates and young children are already well equipped to fight fungal pathogens. The unique response against specific fungi and the relation to the age of the affected organism might paved the way to more specific interventions with less devastating side effects than currently used drugs.

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Author Contributions
K.V. and M.P. designed the study and performed experiments; K.V., A.A., and M.P. performed data analysis; K.V., A.A. and M.C.B.W. wrote the manuscript; K.L., C.A. and D.S. contributed reagents and materials, provided expertise and feedback; M.C.B.W. conceived experiments, received funding, and supervised the study.

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