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Age-related Differences in Immune Reactions to SARS-CoV-2 Spike and Nucleocapsid Antigens

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Abstract. *Background/Aim: The manifestation and severity of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections show a clear correlation to the age of a patient. The younger a person, the less likely the infection results in significant illness. To explore the immunological characteristics behind this phenomenon, we studied the course of SARS-CoV-2 infections in 11 households, including 8 children and 6 infants/neonates of women who got infected with SARS-CoV-2 during pregnancy. Materials and Methods: We investigated the immune responses of peripheral blood mononuclear cells (PBMCs), umbilical cord blood mononuclear cells (UCBCs), and T cells against spike and nucleocapsid antigens of SARS-COV-2 by flow cytometry and cytokine secretion assays. Results: Upon peptide stimulation, UCBC from neonates showed a strongly reduced IFN-γ production, as well as lower levels of IL-5, IL-13, and TNF-α alongside with decreased frequencies of surface CD137/PD-1 co-expressing $CD4^+$ and CD+8 T cells compared with adult PBMCs. The PBMC response of older children instead was characterized by elevated frequencies of IFN-γ+ $CD4^+$ T cells, but significantly lower levels of multiple cytokines (IL-5, IL-6, IL-9, IL-10, IL-17A, and TNF-α) and a marked shift of the $CD4^+/CD8^+$ T-cell ratio towards $CD8^+$ T cells in comparison to adults. Conclusion: The increased severity of SARS-CoV-2 infections in adults could result from the strong cytokine production and lower potential to immunomodulate the excessive inflammation, while the limited IFN-γ production of responding T cells in infants/neonates and the additional higher frequencies of $CD8^+$ T cells in older children may provide advantages during the course of a SARS-CoV-2 infection.*

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Key Words: SARS-CoV-2, COVID-19, spike protein, immune response, inflammation, pediatric immunology, neonates, children.

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The course of a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection varies strongly between affected individuals. Interestingly, the difference in severity of coronavirus-induced disease (COVID-19) appears to depend on the age of the patient (1). Additional host factors including sex and comorbidity are further key determinants of disease severity and progression (2). Apart from the copy number of the infectious agent to which the host was exposed, the immune defense on the mucous membranes and the respiratory epithelium seem to determine the course of disease (3). In some cases, control of the infection is achieved already at that level and without any relevant systemic disease or infection of the lungs. In addition, the ACE-2 receptor plays a central role as an entry portal into the cell and subsequent systemic organ infiltration (4).

When a systemic infection takes place, the control of this process depends on the immune response of the patient, involving all its components from the innate immune system to the cellular and humoral immune response. Up to date, neonates and young children very rarely suffer from a severe course of SARS-CoV-2 infections (5). The responsible factors that contribute to these milder disease courses compared to adults have remained incompletely understood. However, it is widely accepted that the immune responses of neonates, infants, and older children differ in-between ages and profoundly in relation to the one of adults (6-9). By performing *in vitro* studies on specimen from simultaneously

infected family members, we investigated the regulation of immune functions and inflammatory response gained upon exposure of the individuals to SARS-CoV-2.

In a study of complete households with documented SARS-CoV-2 infections, including a cohort of pregnant women, we documented disease symptoms and collected blood from infected adult family members, children of school or kindergarten age, and mature newborns. Heparinized blood, as well as serum samples of adults and children were collected a few weeks after having recovered from SARS-CoV-2 infection, while the samples of the neonates were taken from cord blood directly after birth. In total, we analyzed blood samples from 12 adults, 8 children, and 6 infants/neonates. Peripheral blood mononuclear cells (PBMCs) or umbilical cord blood mononuclear cells (UBMCs) isolated from these blood samples were incubated with either spike or nucleocapsid peptides derived from SARS-CoV-2. The immune responses, in particular the secretion of cytokines, were investigated using ELISpot (IL-10 and IFN-γ secretion), and intracellular cytokine expression was determined by flow cytometric analyses (expression of PD-1, CD137, and IFN-γ of both $CD4^+$ and $CD8^+$ T cells). Further, the accumulation of soluble cytokine levels in the supernatants of the *ex vivo* stimulated cells were determined by a bead-based multiplex assay.

Based on the age-dependent characteristics during childhood compared to adults (6-9), we hypothesized that at different stages of human development, SARS-COV-2 is defended against by different mechanisms. To unravel these ways of defense, we assessed functional parameters, such as cytokine secretion of T cells, and could gain further insight into the more effective immune response in children against SARS-CoV-2 that could be exploited for therapeutic approaches.

Materials and Methods

Subjects and biological samples obtained. We investigated 11 households with a total of 18 adults, 8 affected children older than two years, two infants, and 4 neonates of women who got infected with SARS-CoV-2 during pregnancy (including one set of twins). All of them took part in a longitudinal study of 56 complete households (231 individuals) with one to eight members who had COVID-19 and/or developed antibodies against SARS-CoV-2, focusing on the course of disease, immune responses, and long-term consequences of the infection (ClinicalTrials.gov NCT04741412) (10). This study was approved by the ethics committee of the University Erlangen-Nürnberg and conducted in accordance with the principles of the declaration of Helsinki. All individuals or their legal guardians provided written informed consent to participate.

COVID-19 was diagnosed based on the characteristic symptoms and confirmed by PCR analysis of nasal swabs to detect SARS-CoV-2 or by positive tests for anti-SARS-CoV-2 antibodies. Full blood samples (usually about 2 ml, but approximately 20 ml in case of cord blood) were obtained between November 13th, 2020, and January 4th, 2021. Unfortunately, blood samples from 6 of the 18 adult subjects could not be obtained within this time frame. In total, we collected heparinized blood and additional serum samples from 12 adults (on average 37.4 years old), 8 children (aged 9.1 years on average), two infants (0.15 years old), and 4 neonates on the day of birth.

Preparation of blood samples. Serum was obtained from full blood samples by centrifugation (5 min at 1,000×g). PBMCs, as well as UCBCs, were isolated by density-gradient sedimentation. PBMC and UCBC samples were frozen in cell-freezing medium supplemented with 10% DMSO. Serum levels of anti-SARS-CoV-2 spike antibodies were determined using the NADAL COVID-19 IgG/IgM test (nal von minden GmbH, Moers, Germany) or the Anti-SARS-CoV-2 ELISA (Euroimmun, Lübeck, Germany) according to the manufacturer's instructions.

PBMC activation and ELISpot analysis. PBMCs and UCBCs were thawed and counting of living cells was performed using trypan blue in a cell counting chamber. 0.5×10^5 cells were cultured in X-VIVO 15 medium (Lonza, Basel, Switzerland) supplemented with 4% human AB plasma (Innovative Research, Novi, MI, USA) in 96-well plates. Cells were activated with 200 ng/ml of CEFX Ultra SuperStim Pool (JPT Peptide Technologies, Berlin, Germany), SARS-CoV-2 Peptivator Spike and Nucleocapside peptides (Miltenyi Biotec, Bergisch Gladbach, Germany), or 500 ng/ml PepMix™ Human Actin (JPT Peptide Technologies) in the presence of 500 μg/ml Poly I:C (Biomol, Hamburg, Germany). The cells were then incubated for 48 h, washed, transferred to an ELISpot plate and further incubated for 60 h. ELISpot analysis was performed either with anti-IFN-γ capture and biotinylated detection antibodies (all Mabtech, Stockholm, Sweden) or with the Human IFN-gamma/IL-10 Double-Color ELISPOT Kit (Cellular Technology) according to the manufacturer's instructions; counting took place on an ImmunoSpot analyzer (Cellular Technology, Cleveland, OH, USA). Samples were classified as responders when ELISpot counts were significantly higher than those of negative controls.

Flow cytometric analysis of T cells. A total of 1×10^5 PBMCs or UCBCs were cultured according to ELISpot conditions in X-VIVO 15 medium (Lonza) supplemented with 4% human AB plasma (Innovative Research) in 96-well plates for 6 days. To accumulate cytokines, cells were incubated 4 h prior to flow cytometric analysis with 5 mg/ml brefeldin A. Further, cells were briefly reactivated with 10 ng/ml PMA and 1 μg/ml ionomycin (all Sigma-Aldrich, St. Louis, MO, USA) for 1 h before flow cytometric analysis. After washing, cell surface antigens were stained and then cells were fixed with 2% paraformaldehyde (Morphisto, Offenbach am Main, Germany), permeabilized with 0.5% saponine (Sigma-Aldrich), and subsequently stained for intracellular cytokines. Sample analysis was performed using a FACSCanto II flow cytometer with FACS-Diva software (BD Biosciences, Franklin Lakes, NJ, USA). The following antibodies were used: anti-CD4, anti-CD137 (all Miltenyi), anti-CD3, anti-PD-1, and anti-IFN-γ (all Biolegend, San Diego, CA, USA).

Flow cytometric analysis of cytokines in cell culture media. Levels of IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-22, and TNF-α in cell culture media after 6 days of activation of PBMCs and UMBCs used for flow cytometric analysis were determined using LEGENDplex human Th Cytokine Panel (Biolegend) according to the manufacturer's instructions. Samples were diluted 1:2 in assay

buffer. Measurements were performed with a FACSCanto (BD Biosciences) and LEGENDplex analysis software (Biolegend). Reactivity of all samples was verified by positive controls (CEFX Ultra SuperStim Pool, JPT Peptide Technologies) or heat-inactivated *S. aureus* lysate for neonatal samples above two times the detection limits of analytes (IL-5: 2.5 pg/ml, IL-6: 2.1 pg/ml, IL-9: 0.5 pg/ml, IL-10: 1.3 pg/ml, IL-13: 1.9 pg/ml, IL-17A: 0.3 pg/ml, IL-22: 2.1 pg/ml; TNF-α: 1.5 pg/ml). Unreactive samples were not considered for analysis.

Statistical assessments. Statistical analysis was performed with Prism9 (GraphPad Software, San Diego, CA, USA). Outliers were excluded by Grubbs test (α=0.05). Normal distribution of data sets was tested, and significance analysis was accordingly performed using either parametric or non-parametric tests: two-way ANOVA, Fisher exact test with Freeman-Halton extension, one-way ANOVA (Holm-Sidak's multiple comparisons test), Kruskal-Wallis test with Dunn's correction for multiple comparison, or two-sided Mann-Whitney test.

Results

To determine the immune response of neonates, infants, older children, and adults against SARS-CoV-2 in previously exposed families, nucleocapsid and spike (S1) peptides on the cytokine profile of PBMCs or UCBCs derived from neonates, children and adults, we investigated IL-10 and IFN-γ production by ELISpot and expression of T-cell markers and cytokines by flow cytometric multiplex analyses.

To determine cell-activation of previously exposed members of families, isolated PBMCs from adults and children were loaded with either nucleocapsid or spike peptides of SARS-CoV-2. This resulted in IFN-γ and IL-10 secretion of individual cells in adults and children as detected by ELISpot (Figure 1A). While UCBCs from neonates, as well as PBMCs from infants showed an impaired secretion of IFN-γ as observed in significantly reduced numbers of spots, as well as responding individuals (Figure 1A, B and C), the IFN-γ and IL-10 secretion of individual cells of children appeared to be robust when compared to those of adults (Figure 1B, C and E). Considering the combined spike and nucleocapsid responses to SARS-CoV-2, adult PBMCs showed a bias towards IFN-γ production and their IL-10 responses did not differ from those of children, whereas in neonatal samples only low amounts of IL-10-secreting cells could be detected (Figure 1D and E). Of note, PBMC samples of children did not contain a reduced portion of IFN-γ-secreting cells but exhibited a significantly decreased variance within the individual responses to both spike and nucleocapsid antigens as computed by F test ($p<0.001$) when compared to adults (Figure 1B). Together, the analysis of cytokine secretion of single cells yielded comparable anti-SARS-CoV-2 responses in adults' and children's samples, whereas neonatal cells lacked the capacity to respond – at least in this assay.

To analyze the combined impact of SARS-CoV2 nucleocapsid and spike peptide stimulation on the activation of T-cell subsets of the different age groups, we analyzed the frequencies of CD137 and PD-1 co-expressing cells and the IFN-γ production of both $CD4^+$ and CD+8 T cells by flow cytometry (Figure 2A). Both CD137 and PD-1 can serve as surrogate markers for antigen-specific T-cell activation (11, 12). We found that the ratios of the frequencies of CD137 and PD-1 co-expressing T cells of SARS-CoV-2 peptide activated cells to control cells was significantly reduced in neonatal or infant samples in both $CD4^+$ and $CD8^+$ compartments when compared to those of adults (Figure 2B). PBMCs from children instead showed no difference in the ratios of these T-cells; however, compared to adults, an increased IFN-γ production within the $CD4^+$ T cell subset could be detected (Figure 2B and C). Intriguingly, we found that resting PBMCs of children proportionately harbored significantly more $CD8^+$ T cells and less $CD4^+$ T cells (Figure 2D). Due to the small amount of blood that could be obtained from the umbilical cord, there was not enough material to assess the $CD4^+/CD8^+$ T-cell-ratios in neonatal UCBC cultures. Of note, neonatal cells showed no significant differences in IFN-γ expression of T cells in relation to the other cohorts (Figure 2C). Together, we could further detect age-dependent differences in the capacity to respond to SARS-CoV-2 antigens at the level of T-cell subsets.

To assess age-related differences in further type-1, -2, -9, -17, and -22 secreted cytokines (IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-22, and TNF-α), we analyzed the supernatants of PBMC or UCBC cultures in combined response to SARS-CoV-2 spike and nucleocapsid antigens by a bead-based multiplex assay (LEGENDplex technology). Importantly, stimulation of PBMCs from children showed markedly decreased levels of IL-10 and the pro-inflammatory cytokines IL-6 and TNF-α when compared to adult cell stimulations (Figure 3A and B). Moreover, this effect of children PBMCs could be observed for IL-5, IL-9, and IL-17A except for IL-13 and IL-22 (Figure 3B-D). In infant and neonatal samples, we detected lower levels of IL-5, IL-13, TNF-α, whereas IL-6, IL-9, and IL-10 levels remained similar, when compared to those of adults (Figure 3A-C). Together, the stimulation of PBMCs or UBMCs of adults, children, infants, and neonates, respectively, showed unique signatures of cytokine responses against SARS-CoV-2 antigens. Intriguingly, children PBMCs showed a reduced intracellular cytokine expression of multiple T-helper-cell signatures, such as Th2, Th9, and Th17.

Discussion

This study aimed to identify age-dependent differences in the immune response to SARS-CoV-2 by assessing family members that got simultaneously infected in complete households. Therefore, we investigated not only potential cytokine expression as determined by flow cytometry, but

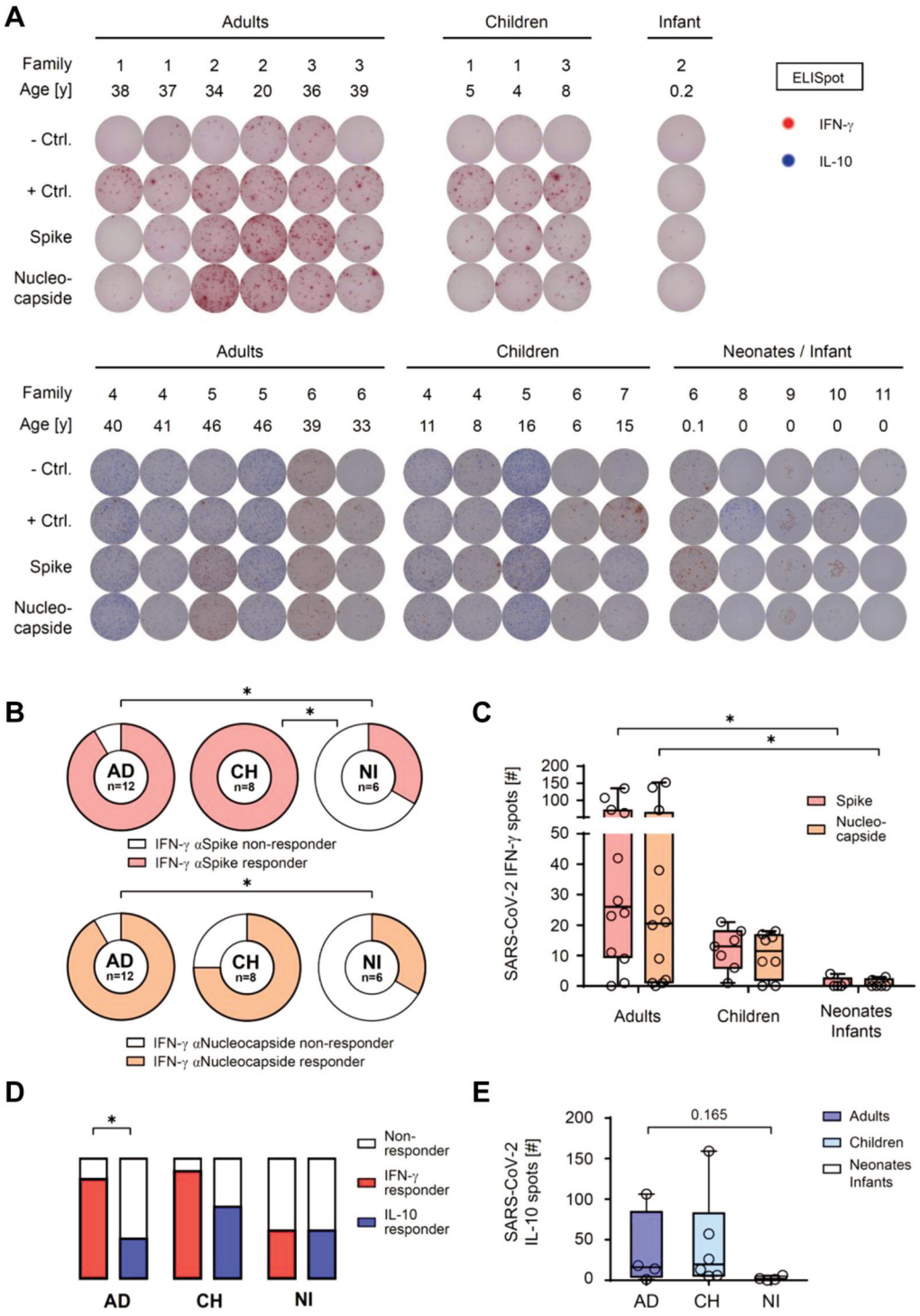

Figure 1. *Augmented IFN-γ response of SARS-CoV-2-infected adults. A) IFN-γ single (upper panel) or IFN-γ and IL-10 double (lower panel) ELISpot scans of peripheral blood mononuclear cell (PBMC) or umbilical cord blood mononuclear cell (UCBC) cultures of SARSCoV-2-infected adults (AD), children (CH), neonates and infants (NI), respectively. Cells were activated with SARS-CoV-2 spike, nucleocapsid, or control peptides. B) Distribution of IFN-γ ELISpot responding or non-responding donors against spike and nucleocapsid peptides according to (A). C) IFN-γ ELISpot counts minus ctrl. of donors reacting to spike or nucleocapsid according to (A). D) Distribution of IFN-γ or IL-10 ELISpot responding or nonresponding donors combined against spike and nucleocapsid peptides according to (A lower panel). E) IL-10 ELISpot counts minus ctrl. of donors reacting to spike and nucleocapsid according to (A lower panel). Data points represent donors with median, interquartile, and range; *p<0.05, p-values were calculated using Fisher exact test with Freeman-Halton extension (B, D), two-way ANOVA (C), or Kruskal-Wallis test (E).*

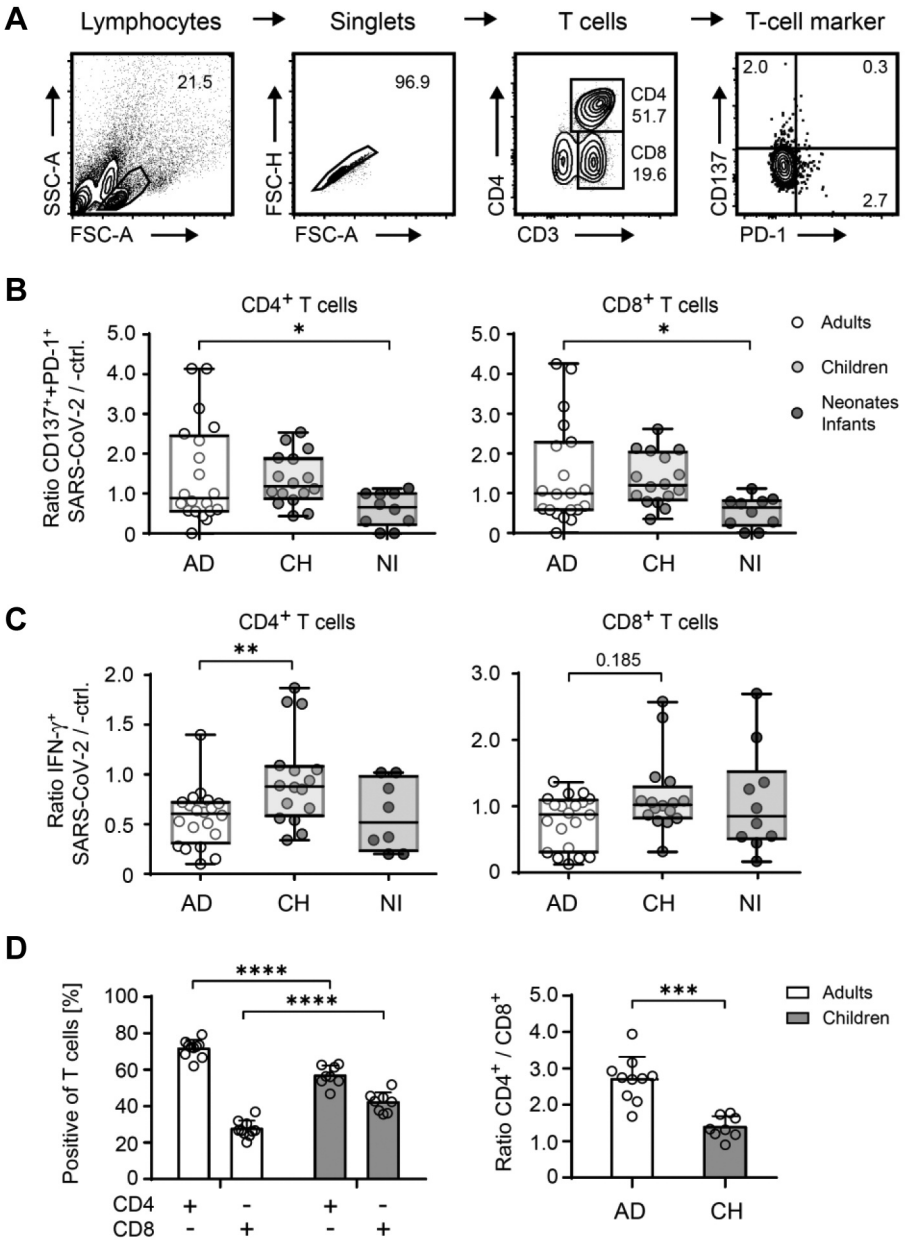

Figure 2. *Flow cytometry analysis of T cells. A) Gating strategy for flow cytometric analysis of T cells. B, C) Ratios of CD137 and PD-1 double-producing (B) or IFN-gamma producing (C) CD4+ (left) or CD8+ (right) T cells in response to SARS-CoV-2 spike and nucleocapside peptides related to negative control. D) Relative amounts of CD4+ or CD8+ subsets of T cells in controls from adult or children peripheral blood mononuclear cells (left) and their ratios (right). Data points represent donors with median, interquartile, and range (B, C) or mean with SD (D); *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; p-values were calculated using one-way ANOVA (Holm-Sidak's multiple comparisons test) (B, C left), Kruskal-Wallis test with Dunn's correction for multiple comparison (C right), two-way ANOVA or two-sided Mann-Whitney test (D).*

also secretion of cytokines of single cells using ELISpot analysis on SARS-peptide-stimulated PBMCs or UCBCs and revealed that adults and children mount robust responses against SARS-CoV-2 antigens but neonates and infants failed to do so. This was most obvious in the detection of IFN-γ and IL-10 secretion of single cells, cytokines that are known to play a central role in the modulation and stimulation of infections (13, 14). As PBMCs or UCBCs were used, detected differences between the age groups could result from variations in the composition of the cell pools. However, in line with other published data, the low IL-10 and IFN-γ production is most likely explained by the low responsiveness

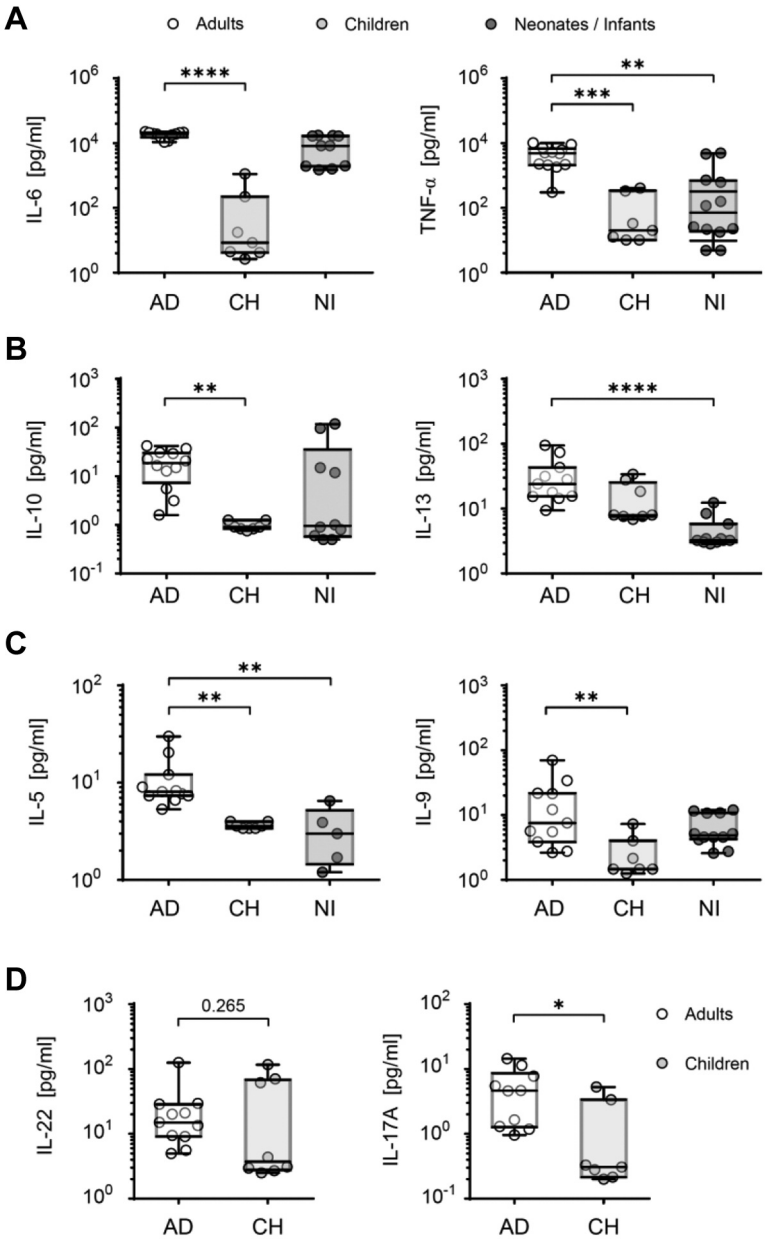

Figure 3. *Augmented cytokine responses of SARS-CoV-2-infected adults compared to children and neonates. A, B) Cytokine levels of IL-6 (A, left), TNF-α (A, right), IL-10 (B, left), and IL-13 (B, right) in the media of peripheral blood mononuclear cell (PBMC) or umbilical cord blood mononuclear cell (UCBC) cultures of SARS-CoV-2-infected adults, children, and neonates, respectively. Cells were activated with SARS-CoV-2 spike and nucleocapside peptides. C, D) Cytokine levels as indicated in cell culture media of PBMCs obtained from donors and activated as in A, B. Data points represent donors with median, interquartile, and range; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; p-Values were calculated using Kruskal-Wallis test with Dunn's correction for multiple comparison (A, B) or two-sided Mann-Whitney test (C, D).*

of the immune system of newborns, with a reduced immune response to pathogens (15). However, contradictory studies using whole heat-inactivated pathogens might be providing additional adjuvant effects than reported here, using re-stimulation with peptides only (6). Our findings are strengthened by further analysis of the neonatal T-cell subsets where both $CD4^+$ and $CD8^+$ T cells showed significantly lower frequencies of CD137+, a costimulatory molecule that is only expressed after the beginning of T-cell stimulation (11, 12), and of PD-1+ cells than adults. The lower frequencies of CD137-expressing cells in neonates implicate reduced proliferation of T-cells and reduced production of pro-

inflammatory cytokines (11). Thus, the impaired responses of neonates and infants could be a result of decreased activation of SARS-CoV-2-specific T cells. Nevertheless, their T cells were able to express IFN-γ protein intracellularly, as detected by flow cytometry, which then failed to be secreted, suggesting that, other than in adults, further signals are necessary to overcome the threshold for cytokine secretion in neonatal T cells. Of note, as the neonatal immune response is known to favor tolerance mechanisms in order to prevent harm to developing tissues, this caution might be an advantage in SARS-CoV-2 defense (16).

When comparing adults with children, the PBMCs of the latter produced more IFN-γ in $CD4^+$ T cells but less overall IL-6, TNF-α, IL-10, IL-5, IL-9, and IL-17A. This enhanced IFN-γ response of children likely reinforces stimulation of bactericidal activity of phagocytes, stimulation of antigen presentation through class I and class II major histocompatibility complex (MHC) molecules, orchestration of leukocyte-endothelium interactions, and likely contributes to effects on cell proliferation and apoptosis (17). Hence, the IFN-γ biased response of children together with less expression of proinflammatory or other T-helper-cell type signature cytokines could lead to improved control and defense against SARS-CoV-2 infections with fewer symptoms caused by an overreacting immune system. The much lower variance in single cell secretion of IFN-γ in comparison to adults could also reflect the appropriately adjusted immune response to SARS-CoV-2 antigens. Intriguingly, under control conditions, children had a significant higher frequency of $CD8^+$ T cells compared to the adults. A subgroup of the $CD8^+$ T-cells are the cytotoxic T-cells, who play an important part in the cellular immune response and aim to stop intracellular (virus) infections by destroying the infected cells through induced apoptosis (18). Thus, the immune response in children could lead to an enhanced clearance of SARS-CoV-2 infected cells and thereby prevent replication, spreading, and exacerbating immune cell activation. In addition, our data on adult samples show a much higher inflammatory reaction triggered by cytokines compared to children and neonates. Previously, a strong cytokine production was discovered as a risk factor for a more severe course in SARS-COV-2 infections (16, 19).

The increased expression and potential release of all cytokines detected in response to SARS-CoV-2 in adults could thus provide an explanation for the strong inflammatory reaction in the lung and the vasculitis, which occur in some adults triggered by a SARS-COV-2 infection. For example, IL-17A is essential for modulating the interplay between commensal microbes and epithelial cells at our borders and yet, for protecting us from microbial invaders, thus preserving mucosal and skin integrity (20, 21). IL-17A takes on a proinflammatory role (22). In patients with psoriasis, IL-17A and IL-17F, which act on immune and non-immune cell types, strongly contribute to tissue inflammation. Psoriasis skin manifestations, cardiovascular, as well as metabolic disease in psoriasis appear to share pathogenic mechanisms evolving around IL-17A and its proinflammatory role. Thus, it was shown that anti-IL-17A therapy in psoriasis patients not only improves skin manifestations of psoriasis, but also cardiovascular inflammation, as well as metabolic factors (23). Moreover, IL-9 is associated with allergic inflammation and mast cell activation (24, 25). Animal data also suggest that pulmonary fibrosis in the context of inflammation may be promoted by IL-9 (26).

In the light of therapeutic intervention in patients with severe COVID-19, this would support the beneficial effects of established immunosuppressive therapy in severe courses of disease, such as dexamethasone (27) or tocilizumab (28, 29).

A further indication of an exacerbating T cell response was the increased proportion of CD137 and PD-1 on both adult $CD4^+$ and $CD8^+$ T cells. Expression of PD-1 on T- and B- cells regulates peripheral tolerance and autoimmunity. Binding of PD-1 to its ligand, PD-L1, leads to protection against self-reactivity (20, 30). CD137 marks the SARS-CoV-2 specifically activated T cells, whereas PD-1 expression has been shown to be necessary to protect against immunopathological side effects during viral infections (31). On one hand, our data suggest that the increased proportion of CD137 and PD-1 co-expressing $CD4^+$ and $CD8^+$ T cells in adults represents their strong reactions to SARS-CoV-2 that need to be counteracted by T-cell co-inhibitory mechanisms. On the other hand, the findings could reflect that adults' T-cell response is already exhausted when taking PD-1 as a surrogate marker for exhaustion. Detection of additional activation-induced molecules on T cells could clarify this issue in future studies.

In summary, the data show in neonates, older children, and adults a different immune reaction to SARS-CoV-2. Children have a higher portion of potentially cytotoxic T-cells, seem to have good T-cell function and more potential to regulate tolerance and terminate inadequate immune response. Adults show an effective immune reaction to spike and nucleocapsid peptides of SARS-CoV-2 with the potential of a cytokine induced overreaction and less potential to immunomodulate the autoimmune inflammation.

As getting informed consent and obtaining blood samples from children and neonates for study reason is challenging, the study group is quite small making the overall statement to be viewed with reservation and should be extended in further studies. Furthermore, it has to be taken into account that cells were obtained from peripheral blood and not from the site of infection. However, in many cases, blood counts correlate with immunological processes as *in vitro* reactivations of peripheral T cells do (11).

Nevertheless, the results of the study address the key question of COVID-19. Why does SARS-COV-2 infections

progress so differently in the individual age groups and what factors contribute to it? The better the understanding of these processes, the more targeted therapeutic approaches and vaccines can be designed. Our study showed in a small cohort significant age-dependent differences in the immune response to spike and nucleocapsid peptides of SARS-COV-2. These differences between children and adults may help to explain why in adults COVID-19 infections might proceed in a more severe way. Whether yet unexplored protective factors are secreted by neonatal and children´s T cells has to be further elucidated.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

P.M., H.S. and H.L. conceived the study, investigated patients, curated data, and wrote the first draft of the manuscript. H.L., S.M. and W.S. performed laboratory investigations. S.K., K.H., A.N., and M.S. collected and evaluated clinical data, supervised by H.S.; M.B.W. curated clinical and laboratory data. All Authors critically reviewed the manuscript, approved its final version and agree to be accountable for all aspects of the work.

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