Activation of human γδ T cells and NK cells by Staphylococcal enterotoxins requires both monocytes and conventional T cells

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Abstract
Staphylococcal enterotoxins (SE) pose a great threat to human health due to their ability to bypass antigen presentation and activate large amounts of conventional T cells resulting in a cytokine storm potentially leading to toxic shock syndrome. Unconventional T- and NK cells are also activated by SE but the mechanisms remain poorly understood. In this study, the authors aimed to explore the underlying mechanism behind SE-mediated activation of MAIT-, γδ T-, and NK cells in vitro. CBMC or PBMC were stimulated with the toxins SEA, SEH, and TSST-1, and cytokine and cytotoxic responses were analyzed with ELISA and flow cytometry. All toxins induced a broad range of cytokines, perforin and granzyme B, although SEH was not as potent as SEA and TSST-1. SE-induced IFN-γ expression in MAIT-, γδ T-, and NK cells was clearly reduced by neutralization of IL-12, while cytotoxic compounds were not affected at all. Kinetic assays showed that unconventional T cell and NK cell-responses are secondary to the response in conventional T cells. Furthermore, co-cultures of isolated cell populations revealed that the ability of SEA to activate γδ T- and NK cells was fully dependent on the presence of both monocytes and αβ T cells. Lastly, it was found that SE provoked a reduced and delayed cytokine response in infants, particularly within the unconventional T and NK cell populations. This study provides novel insights regarding the activation of unconventional T- and NK cells by SE, which contribute to understanding the vulnerability of young children towards Staphylococcus aureus infections.

KEYWORDS
MAIT cell, SEA, SEH, TSST-1, unconventional T cells

1 | INTRODUCTION

Staphylococcus aureus (S. aureus) is considered a commensal bacterium and a frequent colonizer of the human nasopharynx,
skin, and gut. However, _S. aureus_ is also a major cause of nosocomial infections, food poisoning, and sepsis due to production of a wide range of staphylococcal enterotoxins (SE). In addition to causing food poisoning and toxic shock syndrome, certain SE could potentially be weaponized and used in biological warfare.

The virulence of _S. aureus_ is mainly determined by its enterotoxins and 26 SE and SE-like proteins have been described and classified. SE are known to induce polyclonal activation of conventional T cells. This is independent of Ag presentation and potentiated by cross-linking the T cell receptor (TCR) and the MHC class II receptor resulting in proliferation, cytototoxicity and secretion of the cytokines IL-2, IFN-γ, and TNF. Two frequently studied SE, SEA and toxic shock syndrome toxin-1 (TSST-1), mediate conventional T cell activation through several TCR variable _β_-chains (TRBV), while the less studied SEH specifically binds the alpha chain (TRAV27). Notably, although unconventional T cells and NK cells are also readily activated by _S. aureus_ and its SE, the mechanisms involved in their activation by SE still remain poorly understood. Unconventional T cells are non-MHC restricted and include, among others, mucosal associated invariant T (MAIT) cells and γδ T cells. These cell types are described as innate-like lymphocytes that recognize a limited set of microbe-derived antigens and quickly secrete cytokines and act cytopoietic on immune challenge.

MAIT cells are restricted by the MHC class I-like receptor (MR1) and characterized by expression of a semi-variable αδ TCR with a fixed α-chain (TRAV2-1) and high IL-18R and CD161 expression. γδ T cells express a γδ- and δ-chain TCR of which the majority respond to small, phosphorylated bacterial metabolites or lipid-based self-ligands via the receptor butyrophilin 3A1 (BTN3A1) or CD1d, respectively. NK cells are innate lymphocytes lacking a TCR, but capable of clearing infected and transformed cells through cytolytic activities and cytokine production. Although SEA has been reported to directly activate γδ T cells, the binding restrictions of most SE suggest a theoretical inability to directly activate γδ T cells and NK cells, while SEA and TSST-1, but not SEH, could potentially directly activate MAIT cells. On the other hand, these cells are also activated by the presence of inflammatory cytokines in the local environment where APC-derived cytokines are of particular importance. For example, NK cell effector responses are up-regulated by IL-12, IL-15, and IL-18 and IL-12 and IL-18 can induce effector responses in MAIT cells through a TCR-independent mechanism. However, the involvement of innate cytokines in SE-mediated activation of unconventional T cells and NK cells require further investigations.

In the present study, we aimed to investigate underlying mechanisms of SE-mediated activation of MAIT cells, γδ T cells and NK cells in vitro. Further, as _S. aureus_ is one of the leading causes of neonatal sepsis and the immune system of neonates and infants have altered characteristics compared to adults, we also wanted to compare neonatal and adult immune cell responses toward SE.

## 2 MATERIALS AND METHODS

### 2.1 Ethics statement and isolation of cord/peripheral blood mononuclear cells

For the majority of the experiments (Figures 1–4; all Supplementary figures), peripheral blood mononuclear cells (PBMC) from adult volunteers were used, which was approved by the regional Ethics committee at the Karolinska Institute, Stockholm, Sweden. All adult study subjects gave their informed written consent.

In Figure 4, cord blood mononuclear cells (CBMC) were collected from healthy neonates at term deliveries, which was approved by the regional Ethics committee at the Karolinska Institute, Stockholm, Sweden. All mothers gave their informed written consent.

In Figure 5, CBMC/PBMC from healthy children at birth, 2, and 7 years of age, and adults, participating in 2 different cohorts described elsewhere were included. These studies were approved by the Human Ethics Committee at Huddinge University Hospital, Stockholm. According to the regulations present at the time of sample collection, all parents provided informed consent.

Venous or cord blood was collected in heparinized vacutainer tubes (BD Pharmingen, San Diego, CA) and diluted 1:1 with RPMI-1640 supplemented with 20 mM HEPES (HyClone Laboratories, Inc, South Logan, UT). PBMC/CBMC were isolated using Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient separation. Next, cells were washed and resuspended in freezing medium containing 40% RPMI-1640, 50% FBS, and 10% dimethyl sulphoxide (both from Sigma Aldrich, St Louis, MO). The cells were frozen gradually at −80°C in freezing containers (Mr. Frosty, Nalgene Cryo 1°C; Nalge Co, Rochester, NY) and stored in liquid nitrogen.

### 2.2 Production of SEA, SEAD227A, and SEH

The superantigens (SEA, SEH, and SEA-D227A) were produced as earlier described with minor changes. Briefly, untagged superantigens were expressed in _E.coli_ K12 strain UL635. Cells were cultured in 2xYT media supplemented with 90 mM potassium phosphate. At an OD of 0.6, 3% v/v of 30% D-glucose was added and culturing was continued for 18 h. The cultures were collected, and the pellet was flash frozen. The pellets were homogenized in MES pH 5.5 for SEA and SEAD227A and for SEH in NaAc pH 4.0 buffer supplemented with 0.03 mg/mL lysisomine, 2 protease inhibitors cocktail tablets (Roche Diagnostics, Rotkreuz, Switzerland), 1 mM DTT and 1 mM EDTA to collect the protein from the periplasm. The suspension was centrifuged at 180,000 x g for 45 min and the supernatant was diluted 3x in homogenization buffer and was subjected to cation exchange chromatography (Res S 6 ml, GE Healthcare, Chicago, IL) on ÄKTA avant (GE Healthcare). For SEA and SEA-D227A, MES pH 5.5 and for SEH NaAc pH 4.0 buffer were used and the protein was eluted in a gradient from 0 to 1 M NaCl. The superantigens were further purified by size exclusion chromatography.
(Superdex 75 10/300, GE Healthcare) in HBS buffer pH 7.4. Lastly, the superantigens were confirmed to be free of LPS contamination by measuring IL-6 secretion from PBMC upon 24 hours of stimulation. No IL-6 was detected above background levels at concentrations up to 200 ng/mL (Supplementary Fig. S1).

2.3 | In vitro stimulation of CBMC/PBMC

CBMC/PBMC were thawed, washed twice, and viability was assessed by trypan blue staining. Cells were resuspended in cell culture medium (RPMI-1640 supplemented with 20 mM HEPES, 2 mM l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all from HyClone Laboratories Inc.), and 10% FBS (Sigma Aldrich)) to a final concentration of 1 x 10^6 cells/mL. Cells were seeded into cell culture plates and stimulated with 20 ng/mL of SEA, SEH, or TSST-1 (Toxin Technology, Inc., Sarasota, FL) and incubated at 37°C with 5% CO_2_ atmosphere. Cell culture supernatants were collected and stored at −20°C for subsequent ELISA analyses, and cells were analyzed by flow cytometry.

For cellular assays comparing wild-type and mutant SEA-D227A, PBMC were stimulated with wild-type SEA or mutant SEA-D227A at concentrations ranging from 0.002 to 20 ng/mL for 48 h. Cells were analyzed by flow cytometry.

For kinetic experiments, PBMC were stimulated with 20 ng/mL of SEA and incubated for 4, 8, 12, 16, and 24 h.

2.4 | Cell isolation and co-cultures

Monocytes, total CD3⁺ T cells, γδ T cells and NK cells were isolated from PBMC by negative selection using EasySep™ Human monocyte enrichment kit and CD3⁺ T cell-, γδ T cell-, and NK cell isolation kits, respectively (all from STEMCELL Technologies Inc, Vancouver, Canada) according to manufacturer’s instructions. In brief, PBMC were washed and resuspended in PBS supplemented with 2% FBS and 1 mM EDTA (Invitrogen, Waltham, MA) at 50 x 10^6 cells/mL. PBMC were then incubated with an antibody cocktail, followed by incubation with magnetic particles. The cells of interest were recovered by magnetic separation using an EasySep magnet and re-suspended in cell culture medium. For co-culture experiments, 5 x 10⁴ monocytes/well were primed with SEA for 4 h in a V-shaped 96-well plate and subsequently washed carefully by centrifugation. Isolated CD3⁺ T cells (2 x 10⁵ cells/well), γδ T cells (5 x 10⁴ cells/well) or NK cells (5 x 10⁵ cells/well) were added to the SE-primed monocytes in different combinations and incubated for an additional 20 h. Cells were analyzed by flow cytometry.

2.5 | Transwell experiments

Monocytes, total CD3⁺ T cells, γδ T cells, and NK cells were isolated using STEMCELL Technology kits as described above. A total of 1 x 10⁵ γδ T cells or 1 x 10³ NK cells were added to the inserts and 1 x 10⁵ monocytes and 4 x 10⁵ total CD3⁺ T cells were added to the bottom wells of a Costar® 6.5 mm Transwell® 0.4 μm pore polyester plate. SEA was added to the bottom well at a concentration of 20 ng/mL for 24 h at 37°C, 5% CO₂. Cells were then collected and analyzed using flow cytometry. For comparison to membrane separated NK cells: monocytes, total CD3⁺ T cells and NK cells from the same donors were co-cultured in a 48-well plate and stimulated with SEA for 24 h. IFN-γ expression within γδ T cells present among the total CD3⁺ T cells in the bottom well of the transwell plate was used for comparison to membrane separated γδ T cells.

2.6 | Blocking experiments

PBMCs were stimulated in the presence of 2 μg/mL anti-human IL-12 (clone: 24910, R&D Systems Inc, Minneapolis, MN) or 2 μg/mL matched IgG1 isotype control (clone: MOPC-21, BD Biosciences, Franklin Lakes, NJ). In brief, blocking or control antibodies were added to the cells 30 min prior and 24 hours after stimulation with SEA and TSST-1. Cells were analyzed by flow cytometry after a total of 48 h-stimulation.

2.7 | Degranulation assay

Anti-human CD107a V450 antibody (clone H4A3; BD Biosciences) was added to the cells at 0.5 μg/mL immediately prior to stimulation with SEA and TSST-1. Cells were incubated for 48 h and analyzed by flow cytometry.

2.8 | Flow cytometry

For all flow cytometry analyses, except for degranulation experiments, Brefeldin A (GolgiPlug™ BD Biosciences) was added during the last 4 h of stimulation to inhibit protein secretion. After incubation, cells were transferred to 96 well V-shaped staining plates and washed twice in PBS. Cells were stained with LIVE/DEAD Fixable Dead Cell Stain Kit-Aqua (Life Technologies, Carlsbad, CA) or with BD Horizon™ Fixable Viability Stain 780 (BD Biosciences) for 15 min at room temperature. Cell surface Fc receptors were blocked with 10% human serum in FACS-wash buffer (PBS, 2 mM EDTA and 0.1% BSA (Roche Diagnostics) and subsequently stained with the following Abs; CD3- BV510/APC-H7 (clone: UCHT1/SK7), CD28-BV421 (clone: CD28.2), CD56-APC (clone: B159), CD130-BV421 (clone: AM64) (all from BD Biosciences), CD27-PE (clone: M-T271), CD57-PE-Cy7 (clone: HNK-1), CD161-FITC/PE-Cy7/BV421 (clone: HP-3G10), Vz7.2-PE/APC (clone: 3C10), Vz6-APC (clone: B6) (all from BioLegend, San Diego, CA), Pan γδ TCR-FITC (clone: IMMU510), Vz1-PE-Cy7 (clone: R9.12) (both from Beckman Coulter Inc, Brea, CA), and NKG2C-PE (clone: REA205, Miltenyi Biotec, Bergisch Gladbach, Germany). After surface staining, cells were washed and fixed/permeabilized using the Fixation- and Intracellular Staining Permeabilization Wash buffer (BioLegend) according to manufacturer’s instructions. Intracellular blocking was done with 10% human serum followed by staining for intracellular
T- and NK cells occur through non-canonical receptors.

**Prime sequences for RT-qPCR** shows a representative FACS plot for the degranulation staining after SEA stimulation. The levels of IL-2, IL-6, IL-10, IL-17A, IL-21, IFN-γ, TNF, granzyme B, and perforin were quantified in cell culture supernatants using sandwich ELISA according to manufacturer’s instructions (all from MabTech AB, Nacka, Sweden). The optical density was measured using a microplate reader (Molecular Devices Corp, Sunnyvale, CA) at 405 nm wavelength and results were analyzed using SoftmaxPro 5.2 rev C (Molecular Devices Corp.).

**Table 1** Primer sequences for RT-qPCR

| Target gene | Forward primer | Reverse primer |
|-------------|----------------|---------------|
| IL-12       | CCATGGGCTCATGGTGGA | ACGCAGAATGTCAGGAAGAAGAA |
| IL-15       | TGAGAAGCCATCGTGGATG | GTATTGAGAACAGCTGCC |
| IL-16       | GCTGAACCAGTGAAGCATAAGTG | CCAAGTGGTCATCATCTTCAGCTA |
| TBP         | CACGAAACCAGGCCACTGATT | TTTCTTGCTGCAGTCTGGAC |

*TBP: TATA box binding protein.

IFN-γ-PerCP-Cy5.5 (clone: B27), IL-2-BV421 (clone: S344.111) (both from BD Biosciences) or TNF-BV421 (clone: Mab11, BioLegend). Stained cells were washed, resuspended in FACS-wash buffer, and acquired using a FACSVerse instrument and FACS-Suite software (both BD Biosciences). Specific cell populations were gated using forward and side scatter properties followed by fluorescently labelled antibodies. Fluorescence-minus-one and isotype controls were used for gating when appropriate. For complete gating strategy, the reader is referred to Supplementary Fig. S2A and B. Analyses were done with FlowJo software v10.2 (TreeStar, Ashland, OR).

**2.9 | ELISA**

The levels of IL-2, IL-6, IL-10, IL-17A, IL-21, IFN-γ, TNF, granzyme B, and perforin were quantified in cell culture supernatants using sandwich ELISA according to manufacturer’s instructions (all from MabTech AB, Nacka, Sweden). The optical density was measured using a microplate reader (Molecular Devices Corp, Sunnyvale, CA) at 405 nm wavelength and results were analyzed using SoftmaxPro 5.2 rev C (Molecular Devices Corp.).

**2.10 | Reverse-transcriptase qPCR**

PBMC were stimulated for 4 or 8 h after which total cellular RNA was harvested using a RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. From 200 ng of extracted RNA, cDNA transcripts were obtained using SuperScript VILO cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA). RT-qPCR was performed on a LightCycler® 480 II Real-Time PCR instrument (Roche Diagnostics) using gene-specific primers and KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems Inc, Wilmington, MA). Target gene expression was normalized against expression of a housekeeping gene: TATA box binding protein (TBP). Primers were purchased from Eurofins Genomics and primer sequences are shown in Table 1.

**2.11 | Statistics**

Nonparametrical statistical tests were performed on all data sets. Friedman or Kruskal-Wallis tests followed by Dunn’s multiple comparisons were done and Wilcoxon matched-pairs signed rank test was done on comparisons of 2 parameters within the same individual. Analysis and presentation were done using GraphPad Prism (GraphPad Software, La Jolla, CA). All bar and dot plots show medians with interquartile range (IQR) and boxes show the median as the central line with boxes covering the 25th to 75th percentile and whiskers indicating min-max values. Data was considered significant if \( P < 0.05 \) and the following significance levels were used: \( *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 \).

**2.12 | Online supplemental material**

Figure S1 shows IL-6 secretion upon stimulation with in-house produced SE. Figure S2 shows a complete gating strategy used for flow cytometric analyses. Figure S3 shows the proportion of CD130 expressing cells within unconventional T- and NK cells at baseline and after stimulation. Figure S4 shows a representative FACS plot for the activation kinetics of T- and NK cells. Figure S5 shows a representative FACS plot for the degranulation staining after SEA stimulation.

**3 | RESULTS**

**3.1 | SE-mediated activation of γδ T- and NK cells is dependent on physical contact with monocytes and conventional T cells**

Although structurally and functionally similar, SEA, SEH, and TSST-1 all differ in their binding orientation and specificities toward the MHC class II and TCR. If and how this affects the subsequent immunological response is largely unknown. Following PBMC-stimulation with these three toxins, all cell types investigated – conventional T cells, γδ T cells, MAIT cells, and NK cells – were induced to express IFN-γ, although SEH was comparably less potent (Fig. 1A). Conventional T cells were clearly activated at lower toxin concentrations compared to the other cell types investigated. In addition, following stimulation with a SEA-D227A mutant toxin, which is unable to bind MHC class II, the IFN-γ production was significantly reduced in all investigated cell types at comparable concentrations (Fig. 1B).

γδ T- and NK cells lack the canonical receptors for superantigen-TCR interaction. Still, SEA also interacts with, and promote signaling through, the IL-6 co-receptor CD130 on adipocytes. However, we could not detect expression of CD130 on NK cells or γδ T cells (Supplementary Fig. S3). In order to further investigate whether SE-mediated activation of γδ T- and NK cells occur through non-canonical receptors, we developed a co-culture system where isolated γδ T- or NK cells
FIGURE 1  Indirect activation of γδ T- and NK cells by staphylococcal enterotoxins. Stimulated PBMC were stained for intracellular cytokine expression and analyzed using flow cytometry. (A) Proportion of IFN-γ+ conventional (Conv.) T-, γδ T-, MAIT-, and NK cells after 24 and 48 h of stimulation with SEA, TSST-1 or SEH, (n = 10). (B) Proportion of IFN-γ+ cells upon stimulation with wild-type or mutant SEA-D227A, (n = 6-8). The level of significance between wildtype and mutant SEA-D227A for each concentration is shown. (C) IFN-γ expression within NK cells, (n = 6-7) and γδ T cells, (n = 3-6), upon 20 h of stimulation of purified cells or of co-cultures with isolated monocytes, total CD3+ T cells or monocytes + total CD3+ T cells combined. (D) IFN-γ expression within NK cells, (n = 6) and γδ T cells, (n = 6), upon 24 h of stimulation in membrane separated transwell plates. (E) Proportion of IL-2+ and IFN-γ+ Conv. T-, γδ T-, MAIT- and NK cells after 4, 8, 12, 16 and 24 h of stimulation with SEA, (n = 6-8). a (*) and b (**) represent levels of significance between stimulated and unstimulated samples for each cell type and timepoint. Dot-plots show median with interquartile range, box plots show median as the central line with boxes covering the 25th to the 75th percentile and whiskers represents min-to-max
A

**FIGURE 2** Involvement of innate-derived cytokines in SE-mediated activation of T- and NK cells. (A) SE-stimulated PBMC were analyzed for transcriptional activation of IL-12, IL-15 and IL-18 by reverse-transcriptase qPCR after 4- and 8 h of stimulation, \((n = 4)\). (B) PBMC were stimulated in the presence of an anti-IL-12 neutralizing Ab or isotype control, \((n = 4-7)\), where the proportion of IFN-\(\gamma^+\) (top row) or CD107a\(^+\) cells (bottom row) among each cell type was normalized to the isotype control, which was set to 1. (C) Secretion of Perforin and Granzyme B after anti-IL-12 neutralization was measured with ELISA, \((n = 4-6)\). Bar plots show median with interquartile range.

were added to SE-primed autologous monocytes followed by analyses of intracellular cytokine responses using flow cytometry. Interestingly, none of the SE were able to induce IFN-\(\gamma\) in pure \(\gamma\delta\) T- or NK cell cultures, nor were SE-primed monocytes able to induce IFN-\(\gamma\) expression in autologous \(\gamma\delta\) T- or NK cells (Fig. 1C). However, addition of total CD3\(^+\) T cells to either co-culture systems did result in activation of both \(\gamma\delta\) T- and NK cells (Fig. 1C), suggesting that SE-induced activation of these cells requires the presence of both \(\alpha\beta\) T cells and monocytes. In order to determine whether cell-to-cell contact was required for activation, \(\gamma\delta\) T- and NK cells were seeded into inserts of 0.4 \(\mu\)m pore membrane transwell plates with SEA-stimulated monocytes and total CD3\(^+\) T cells in the bottom well. In the absence of physical contact, \(\gamma\delta\) T- and NK cell derived IFN-\(\gamma\) was lost (Fig. 1D), suggesting an important role for surface-expressed co-stimulatory receptors. In addition, conventional T cells expressed IL-2 and IFN-\(\gamma\) already after 4 hours of stimulation whereas NK-, \(\gamma\delta\) T-, and MAIT cell-activation required longer durations of stimulation (Fig. 1E; Supplementary Fig. S4). Collectively, this indicates that SE-mediated activation of \(\gamma\delta\) T- and NK cells occur through an indirect mechanism dependent on the presence of monocytes and conventional T cells.

3.2 | SE-induced IFN-\(\gamma\) expression in T- and NK cells is IL-12 dependent

To study the involvement of APC-derived cytokines in SE-mediated activation, we isolated RNA from stimulated PBMC and analyzed the expression of IL-12, IL-15, and IL-18 using RT-qPCR. We observed an up-regulation of IL-12 transcription by all SE while IL-15 and IL-18 levels remained unchanged at both timepoints investigated (Fig. 2A). Next, PBMC were stimulated with SEA or TSST-1 in the presence of an anti-IL-12 neutralizing Ab or isotype control and IFN-\(\gamma\) expression and cytotoxic granule release were analyzed using flow cytometry. For both SE, neutralizing IL-12 reduced the frequency of IFN-\(\gamma\) expressing
FIGURE 3 Phenotypical characterization of IFN-γ responses in γδ T- and NK cells. PBMC were stimulated for 48 hours and the association between surface markers and IFN-γ production was evaluated in different NK cell- and γδ T cell sub-populations using flow cytometry. (A) IFN-γ induction by SEA, TSST-1 and SEH among TRDV1 +, TRDV2 +, or TRDV1-/V2- γδ T cells, (n = 7-11). (B) Proportion of IFN-γ+ cells among CD27+/−, CD28+/−, or CD161+/− TRDV2 + γδ T cells in response to SEA stimulation, (n = 6-8). (C) Proportion of IFN-γ+ cells among CD57+/−, NKG2C+/−, or CD161+/− NK cells in response to SEA stimulation, (n = 8-12). Box plots show median as the central line with boxes covering the 25th to the 75th percentile and whiskers represent min-to-max.

3.3 δ-Chain usage and phenotypic markers associate with SE-induced IFN-γ expression in γδ T cells

γδ T cells are commonly subdivided according to Vδ-chain usage, which also correlates with tissue distribution and function where TRDV2+ cells are more frequently found in circulation and TRDV1/3+ at mucosal sites.11,30 We first investigated IFN-γ expression in TRDV1+, TRDV2+, and TRDV1− TRDV2− γδ T cells upon SE stimulation of PBMC using flow cytometry (Supporting information Fig. S2B). All three γδ T cell subsets expressed IFN-γ upon stimulation and we observed that SEA and TSST-1 induced a stronger response compared to SEH within each subset (Fig. 3A).

In the circulation, the TRDV2+ subpopulation is the most prevalent among γδ T cells. We therefore investigated whether SE-induced responses in TRDV2+ T cells associated with a particular phenotype. We specifically investigated the memory-associated markers CD27, CD28, and CD161, commonly associated with pro-inflammatory responses in memory T cells.31 Both CD27- and CD28 expression correlated with increased IFN-γ expression upon SEA stimulation (Fig. 3B). Also, we have previously shown that SEA induces up-regulation of CD161 followed by increased...
production of pro-inflammatory cytokine expression in CD4+ FOXP3+ T cells. However, we observed no significant association between IFN-γ and CD161 expression within the TRDV2+ T cell population (Fig. 3B).

CD57 and NKG2C mark NK cells of a more differentiated state where NKG2C+ NK cells associate with increased IFN-γ production during human cytomegalovirus infection. However, we observed no correlation between IFN-γ expression and surface expression of the markers CD57 or NKG2C among NK cells in SEA-stimulated PBMC cultures (Fig. 3C). The relationship between CD161 expression and NK cell activity has been debated. In our current setting, there was no association between IFN-γ- and CD161 expression among NK cells (Fig. 3C).

3.3 The response to SE is delayed and weaker in newborns compared to adults

In order to investigate the response to SE at different stages of immune maturation, we analyzed a set of cytokines (IL-2, IL-10, IL-17A, IL-21, IFN-γ, and TNF) and cytotoxic compounds (granzyme B and perforin) released by CBMC and adult PBMC upon stimulation using ELISA. Twenty-four hours after stimulation, only IL-2 was detected in CBMC supernatants, whereas all the other factors were detected in adult PBMC supernatants (Fig. 4A). After 48 h, CBMC secreted detectable levels of IL-2, IFN-γ, TNF, granzyme B, and perforin, but still in lower amounts compared to adult PBMC (Fig. 4B). IL-10, IL-17A, and IL-21 were not detected or barely detected in cord blood after 48 h of stimulation, although readily secreted by adult PBMC (Figs. 4A, B). Overall, and in accordance with our other findings, SEA induced the highest levels of secreted cytokines and cytotoxic compounds, and SEH the lowest levels, in both CBMC and PBMC, with the exceptions of IL-10 and IL-17A (Figs. 4A, B).

3.4 SE do not induce IFN-γ in unconventional T cells and NK cells during the neonatal and infant period

We next investigated the activation of different T-cell populations and NK cells at several time points during early childhood by stimulating CBMC or PBMC from 2- and 7-year-old children and adults with SEA or TSST-1 for 48 h.

First, we assessed the frequency of each cell type in absence of stimulation (Fig. 5A). The highest frequency of MAIT cells was found in adults but was barely detectable in cord blood, which is in accordance with previous studies. γδ T cells were detected in all age groups, and at the highest frequency in 2-year-olds, while NK cells were found in highest frequency in cord blood (Fig. 5A). In terms of the response to SE, conventional T cells from all age groups produced IFN-γ upon stimulation with SEA and TSST-1, with the highest expression noted in adult cells (Fig. 5B). MAIT-, γδ T- and NK cells in cord blood or from 2-year-old children failed to produce IFN-γ in response to the toxins. However,
FIGURE 5  The IFN-γ production in response to SE is diminished early in life. CBMC and PBMC from 2-year-olds, 7-year-olds and adults were stimulated with SEA or TSST-1 for 48 h. (A) Frequencies of MAIT-, γδ T- and NK cells found among lymphocytes from cord blood (CB), (n = 6), 2-year-olds, (n = 7), 7-year-olds, (n = 7), and adults, (n = 8), in unstimulated samples. (B) The proportion of IFN-γ+ cells within conventional (Conv.) T-, MAIT-, γδ T- and NK cells in CB, (n = 6), 2-year-olds, (n = 7), 7-year-olds, (n = 7) and adults, (n = 8), upon stimulation. (C) SEA-induced IL-12 transcription after 8 hours of stimulation in PBMC from 2- and 7-year-olds (n = 5-6). The level of significance between stimulated and unstimulated samples within each age group is shown. Bar plots show median with interquartile range and symbols represent individual values in the samples from 7-year-olds, MAIT-, γδ T-, and NK cells significantly responded to both toxins.

Since we observed a significant role of IL-12 production for efficient IFN-γ responses in adult cells, we stimulated PBMC of 2- and 7-year-olds with SEA for 8 h and analyzed IL-12 induction by RT-qPCR. Interestingly, SEA-induced IL-12 transcription was clearly hampered in both 2- and 7-year-olds, (Fig. 5C) compared to adults (Fig. 2A). Altogether, we have shown that unconventional T- and NK cells from cord blood and 2-year-old children failed to produce IFN-γ in response to SE, and that SEA-induced IL-12 transcription was lower in early life.
4 | DISCUSSION

Several reports have shown that unconventional T cells and NK cells can be activated by bacterial toxins, although the mechanisms and physiological consequences are only just beginning to be revealed. \(^7,8,37,38\) We have previously shown that SEA induces IFN-\(\gamma\) production in MAIT-, \(\gamma\delta\) T-, and NK cells in a partly IL-12 dependent manner. \(^5\) Here, we have used the toxins TSST-1 and SEH, as well as SEA, to further investigate the mechanisms involved in the activation of unconventional T cells and NK cells by SE. We report that MAIT-, \(\gamma\delta\) T-, and NK cells all produced the proinflammatory cytokine IFN-\(\gamma\) toward SEA, TSST-1, and SEH, and that SE-induced activation of \(\gamma\delta\) T- and NK cells required physical cell contact with \(\alpha\beta\) T cells and monocytes. Furthermore, IL-12 neutralization inhibited SE-induced IFN-\(\gamma\) expression while cytotoxic granule release remained unaffected. Finally, we found that the production of cytokines as well as cytolytic compounds in response to SE-stimulation was absent or diminished in the first years of life.

It is well established that superantigens activate conventional T cells by crosslinking TCR TRBV-chains (SEA, TSST-1) or TRAV27 (SEH) with the MHC class II receptor. \(^5\) The MAIT cell TCR is comprised of the TRAV1-2 chain paired with a limited number of TRBV-chains, thus some MAIT cells could carry a potential binding site for SEA and TSST-1. In theory, \(\gamma\delta\) T- and NK cells are less likely to be directly activated by SE since they do not express an appropriate \(\alpha\beta\) TCR. However, we here observe that these cell types express IFN-\(\gamma\) upon SE-stimulation of PBMC. SEH was clearly less potent in activating unconventional T cells and NK cells as compared to SEA and TSST-1 when used at similar concentrations. It is reasonable to assume that the number of TCR V-chains the SE can engage would correlate with the proportion of responding T cells and the amount of cytokine secretion. Of the three SE used in this study, SEA can bind the greatest number of V\(\beta\)-chains, and is also the only included toxin containing two separate binding sites for the MHC class II receptor. \(^5\) This results in the ability to cross-link multiple MHC class II molecules on the monocyte. \(^39\) We also report that higher concentrations of the mutant SEA-D227A are required in order to induce the same level of activation as wild-type SEA. The fact that SEA-D227A binds to MHC class II with a much-reduced affinity, highlights the importance of this interaction to activate also unconventional T cells and NK cells. Indeed, SEA tended to induce the highest frequency of IFN-\(\gamma^+\) cells and the highest levels of secreted IFN-\(\gamma\) compared with the other toxins used.

Despite the fact that \(\gamma\delta\) T- and NK cells are unlikely to be direct targets for SE, reports suggest alternative modes of activation that do not rely on crosslinking between the \(\alpha\beta\) TCR and MHC class II. SEA has been shown to activate T cells in a TCR-independent manner by binding to the laminin receptor-subunit LAMA2. \(^40\) Furthermore, SEA binds to the IL-6 co-receptor CD130 and activates STAT3 signaling in human adipocytes \(^29\) and a binding site for SEA on the \(\gamma\delta\) TCR has been reported. \(^19\) However, the proportion of unconventional T cells and NK cells expressing CD130 was negligible both at baseline and upon stimulation. \(S.\) aureus infected monocyte-derived dendritic cells specifically activate TRDV2\(^+\) \(\gamma\delta\) T cells in co-culture models, which is dependent on both IL-12 production and surface expressed receptors including the \(\gamma\delta\) TCR. \(^41\) However, by performing co-culture experiments with isolated cell populations, we here demonstrate that activation of \(\gamma\delta\) T- and NK cells by SEA was dependent on both monocytes and total CD3\(^+\) T cells in a contact-dependent manner, but also that IL-12 was necessary for the activation of these cells. We also observed a marked IFN-\(\gamma\) response in TRDV1\(^+\) and TRDV1/2\(^-\) subpopulations in addition to TRDV2\(^+\) \(\gamma\delta\) T cells, further highlighting key mechanistic differences between live bacterial infections and SE-induced activation of \(\gamma\delta\) T cells. We also investigated the kinetics of cell activation upon SE-stimulation and show that conventional T cells are the first to respond, further indicating that the activation of MAIT-, \(\gamma\delta\) T-, and NK cells is secondary to the response elicited by conventional T cells. Taken together, these results suggest an indirect mechanism of activation and an indispensable role for \(\alpha\beta\) TCR expressing T cells in the activation of unconventional T- and NK cells. A similar outcome was observed in an in vivo study in mice, showing that SEA-mediated activation of \(\gamma\delta\) T- and NK cells was dependent on \(\alpha\beta\) T cells. \(^42\)

APC-derived cytokines such as IL-12, IL-15, and IL-18 are known to regulate survival, cytotoxic activity, and IFN-\(\gamma\) responses of T and NK cells. \(^33,43–45\) Furthermore, MAIT cells can be indirectly activated in a TCR-independent manner by IL-12 and IL-18. \(^21,46\) Here, we demonstrate that SE induced a notable upregulation of IL-12 but not of IL-15 or IL-18. Upon IL-12-neutralization, we observed a significant decrease in SE-induced IFN-\(\gamma\) expression in all cell types studied. This was expected since IL-12 is the main driver of Th1 differentiation. In opposite, cell-specific degranulation (CD107a surface expression) and the secreted levels of cytotoxic compounds (granzyme B and perforin) from PBMC-cultures were not significantly affected by IL-12-neutralization. Type 1 IFN are known to be important, especially for cytotoxic activity in NK cells, \(^47\) and \(S.\) aureus have been shown to induce IFN\(\gamma\) in both mice and humans. \(^48,49\) Thus, it would be relevant to further investigate the induction of type 1 IFN upon stimulation with SE and its potential involvement in SE-induced cytotoxicity. In summary, we have shown that monocyte-production of IL-12 is crucial to activate conventional T cells, which in turn are required to activate MAIT-, \(\gamma\delta\) T- and NK cells to produce IFN-\(\gamma\).

Further characterization of \(\gamma\delta\) T cells and NK cells after activation by SE demonstrated that responding \(\gamma\delta\) T cells showed a memory-like phenotype whereas no such associations were found in NK cells by the approach used in this study. SE-induced IFN-\(\gamma\) in \(\gamma\delta\) T cells correlated with CD28 expression. Indeed, several SE, including SEA, SEB, and TSST-1, have been shown to bind to CD28, a surface molecule required for induction of SE-mediated lethal cytokine storm in mice. \(^50,51\) Although the CD28 binding site is relatively conserved among different SE, \(^52\) it is possible that differences in CD28 binding also contribute to the observed weaker cytokine responses induced by SEH compared with SEA and TSST-1. CD161 is a C-type lectin-like receptor expressed on NK cells as well as on several T cell subsets. CD161 expression associates with a high propensity for cytokine production in T cells, \(^32,55,53\) while its functional role in NK cell activity is less clear. Studies report CD161-mediated inhibition of NK cell cytotoxicity \(^54,55\) as well as increased NK cell responsiveness. \(^56\)
Here, we observed no difference in IFN-γ expression between CD161-negative and -positive NK cells or TRDV2+ γδ T cells. CD161 expression correlates with increased expression of the IL-18Ra on both conventional and γδ T cells. Moreover, stimulation of these cells with IL-12 + IL-18 results in production of IFN-γ mainly within the CD161+ γδ T cells. SE failed to induce IL-18 transcription, and IL-18 neutralization had no effect on IFN-γ expression (data not shown), which could explain the observed lack of correlation between CD161 and IFN-γ expression among NK and γδ T cells in our study.

Throughout this study, we have demonstrated that SE activate adult unconventional T cells and NK cells, and that these seem to contribute to the bulk of IFN-γ production. To determine if these responses were influenced by age and level of immune maturation, we investigated the cytokine profile and cellular responses upon stimulation with SE at several time-points during early childhood. NK cells have been reported to be more abundant in cord blood than in adults, which is in agreement with our results. In addition to being more abundant, cord blood NK cells seem to mount a strong IFN-γ response when stimulated with IL-12 and IL-18. Also γδ T cells have been shown to be potent in early life and possess a mature phenotype and a level of response similar to adults already at 2 years of age. Still, neither MAIT-, γδ T- nor NK cells responded with IFN-γ production to any of the SE in the samples from cord blood or 2-year-olds. Moreover, production of a broad range of cytokines and cytotoxic compounds was markedly reduced and delayed from stimulated cord blood compared with adult cells, implying that towards certain stimuli, such as SE, cytokine responses from cord blood and young children are indeed impaired. Conventional T cells of all age groups produced IFN-γ in response to SEA and TSST-1, however the proportion of IFN-γ-producing cells notably increased with age, possibly related to different proportion of memory cells in children and adults. In addition, increased APC capacity to secrete IL-12 in cord blood, and the Th2 bias early in life might be contributing factors to the lower responsiveness observed in neonates and young children. Previous studies suggest that the lower levels of IL-12 mRNA are not due to decreased transcription but to a lower stability of the transcript. Here, we assessed IL-12 transcription in 2- and 7-year-olds, which was clearly reduced in response to SEA compared to adults. Altogether, this suggests that the relative unresponsiveness of unconventional T cells and NK cells during infancy could be a consequence of reduced conventional T cell activation due to a decreased APC response.

In this study, we conclude that several SE, with different TCR V-chain specificities, are able to induce IFN-γ expression in MAIT cells, γδ T cells and NK cells and suggest that their activation is secondary to and requires the presence of γδ TCR-expressing T cells, APC and IL-12. Importantly, we confirm that neonatal T- and NK cells have a reduced capacity to mount a response towards SE in vitro compared with adults. S. aureus is a major contributor to hospital and community acquired infections frequently causing severe conditions such as toxic shock and sepsis. Unconventional T cells have a clear role in these life-threatening conditions and elucidating the mechanism behind their involvement is important.

Our study expands the understanding of the mechanisms involved in SE-induced activation of unconventional T cells and NK cells and suggests a possible contributor to the vulnerability to infections observed during the neonatal and infant period.

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DISCLOSURE
The authors declare no conflict of interest.

AUTHORSHIP
M.M.F., K.L.P., S.B., and E.S.E. were responsible for the conceptualization. M.M.F., C.A., W.v.Z., S.U., A.S., P.R., J.S., K.L.P., and S.B. took part in the investigation and M.M.F., C.A., S.B., and E.S.E. were responsible for data curation, writing of the original draft, editing, and revision.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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