Di-(2-ethylhexyl)-phthalate interferes with T-follicular helper cell differentiation and cytokine secretion through signaling lymphocytic activation molecule family member-1

Yu Han\textsuperscript{a}, Xiaoying Wang\textsuperscript{b}, Xiaoxiao Pang\textsuperscript{b}, Mangze Hu\textsuperscript{b}, Ying Lu\textsuperscript{c}, Jianhua Qu\textsuperscript{a} and Gang Chen\textsuperscript{a}

\textsuperscript{a}Department of Occupational Medicine and Environmental Hygiene, College of Public Health, Nantong University, Nantong, China; \textsuperscript{b}Department of Immunology College of Medicine, Nantong University, Nantong, China; \textsuperscript{c}Department of Nutrition and Food Hygiene, College of Public Health, Nantong University, Nantong, China

\section*{ABSTRACT}

Exposure to the widely-used phthalate plasticizer di-(2-ethylhexyl)-phthalate (DEHP) has been shown to be closely related to an increased prevalence of allergic diseases in infants and juveniles. Earlier work in our laboratory found that DEHP-related anaphylactic responses could be ascribed to T-follicular helper (T\textsubscript{fh}) cell hyperfunction directly. The T\textsubscript{fh} cell, a newly identified CD4\textsuperscript{+} T\textsubscript{cell} subset, until recently has been considered as a key player in humoral immunity. T\textsubscript{fh} cells can respond to stimulation through various receptors. Signaling lymphocytic activation molecule family member-1 (SLAMF1, CD150) is a surface co-stimulatory receptor that can bind to an intracytoplasmic adaptor signaling lymphocytic activation molecule-associated protein (SAP) to initiate downstream signaling cascades, regulating some events of immune response. The present study explored the role of SLAMF1 in T\textsubscript{fh} cell differentiation and cytokine secretion under the condition of DEHP exposure. Using a weanling mice model of DEHP gavage with ovalbumin (OVA) sensitization, it was found that DEHP acted as an immunoadjuvant to elevate SLAMF1 and SAP expression in host T\textsubscript{fh} cells. \textit{Ex vivo} studies of effects from DEHP exposure on T\textsubscript{fh} cells from OVA-sensitized hosts showed that DEHP acted in an adjuvant-like manner to promote the expression of adaptor protein SAP, transcription factors Bcl-6 and c-MAF, and cytokines interleukin (IL)-21 and IL-4 in T\textsubscript{fh} cells. Transfection of these T\textsubscript{fh} cells with Slamf1 small interfering RNA prior to exposure to the DEHP attenuated the over-expression of these molecules that was caused by the DEHP. In conclusion, this study demonstrated that DEHP, via a SLAMF1-mediated pathway, can impact on T\textsubscript{fh} cell differentiation and their ability to form select cytokines.

\section*{Introduction}

Di-(2-ethylhexyl)-phthalate (DEHP) is a commonly-used phthalate plasticizer to impart flexibility to polyvinyl chloride (PVC) products such as food packaging, bottles, toys, building materials, and medical devices. Due to its wide application and stability of its chemical structure, as well as its non-covalently binding to/ easily leaching from PVC, DEHP is increasingly being detected in soil, water, and atmospheric dust particles. DEHP is considered an environmental persistent organic pollutant, and its potential impact on human health has been highlighted on a global scale (Erythropel et al. 2014; Gao and Wen 2016). Human exposure routes to DEHP are multiple, and include inhalation, skin and medical contact, as well as ingestion of contaminated foods, waters, and other materials (Johns et al. 2015). In particular, children are more likely to be exposed to DEHP by sucking or chewing on plastic products and so, consequently, they are more susceptible to potential DEHP hazards (Braun et al. 2013; Wang et al. 2018). Once DEHP is absorbed in the digestive tract, it enters the hepato-enteral circulation and is rapidly metabolized to mono-2-ethylhexyl-phthalate (MEHP) and other secondary products in the liver (Wittassek and Angerer 2008).

Recent epidemiology surveys have noted a positive association between DEHP exposure and increased prevalence of anaphylactic diseases, including allergic rhinitis, bronchial asthma, and atopic dermatitis among genetically-stable populations, especially infants and juveniles (North et al. 2014; Wang et al. 2014; Beko et al. 2015; Li et al. 2017). Anaphylaxis is a hypersensitivity reaction rooted in an imbalance in host humoral immunity (Galli and Tsai 2012; Holgate 2012). A previous study from our laboratory showed that gavage with DEHP resulted in improper enhancement of humoral immune responses (re: to ovalbumin [OVA]) among OVA-sensitized weanling mice. DEHP acted as an immunoadjuvant to augment OVA-specific IgE and IgG1 production, amplified germinal center formation in lymphoid nodules, as well as stimulated expansion of T-follicular helper (T\textsubscript{fh}) and plasma cells. Based on results of immune adoptive transfusion, that study also found these outcomes were related to intrins ic dysfunction among host T\textsubscript{fh} cells (Han et al. 2014). The T\textsubscript{fh} cell is a new subset of CD4\textsuperscript{+} T-helper (T\textsubscript{H}1) cells; until recently, T\textsubscript{fh} cells had been identified as the most important subpopulation to potently assist B-cells and so mediate humoral immune responses (Johnston et al. 2009; Nurieva et al. 2009).
Tfh cells retain high levels of expression of the chemokine receptor CXCR5; this functionally directs them toward B-cell follicular areas via chemotactic effects from the specific ligand CXCL13. Tfh cells synthesize characteristic cytokines (such as interleukin [IL]-21 and IL-4) that facilitate antibody production by and class-switch recombination events in B-cells. Over recent years, it has been shown that the expression of typical transcription factors (such as Bcl-6 and c-MAF) – along with their mutual regulation – is of crucial significance for the initial differentiation of, and essential functions thereafter, of Tfh cells (Crotty 2014; Qi et al. 2014; Butler and Kulu 2015; Jogdand et al. 2016; Wali et al. 2016; Qin et al. 2018).

Like most T-cells, Tfh cells can respond to stimulation through various receptors (Webb and Linterman 2017). The signaling lymphocytic activation molecule (SLAM) family of receptors belong to a CD2 subgroup of the immunoglobulin (Ig) superfamily, with a series of SLAM isoforms exhibiting diverse cellular distributions and functional properties. Among the isoforms, SLAM family member-1 (SLAMF1, CD150) is the prototype that is constitutively expressed on the surface of T-and B-cells, dendritic cells, monocytes-macrophages, natural killer (NK) cells, and NK-T cells (van Driel et al. 2016; Wu and Veillette 2016; Dragovich and Mor 2018). SLAMF1 possesses one or more copies of an immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic tail, through which it recruits Src homology 2 (SH2) domain-containing signal transduction molecules like SLAM-associated protein (SAP, encoded by gene Sh2d1a) to initiate downstream signaling cascades (Vilar et al. 2011; Romero et al. 2014). Thus, SLAMF1 may play an important role in regulating co-stimulatory signals, immunological synapse formation, cytokine synthesis, high-affinity antibody production, and other key properties in immune cells (Yusuf et al. 2010; Zhao et al. 2012; Zhong and Veillette 2013a, 2013b).

To obtain a better understanding of how DEHP might have induced the earlier-noted effects in Tfh cells, and to better explore any role of SLAMF1 in Tfh cell differentiation and cytokine secretion (under the condition of DEHP exposure), the present study examined the influence of DEHP on SLAMF1-SAP-dependent signal transduction pathways in Tfh cells. It was hoped that the data from this study could provide a new understanding of DEHP immunotoxic effects and the mechanisms underlying such outcomes.

## Materials and methods

### Chemicals

DEHP (purity ≥99.5%), OVA (purity ≥98%), and dimethyl sulfoxide (DMSO, purity ≥99.5%) were purchased from Sigma (St. Louis, MO). Slamf1 small interfering RNA (Slamf1 siRNA, ID: 71714) and negative control siRNA (NC siRNA) were purchased from Thermo Fisher Scientific (Waltham, MA). Anti-mouse CD3 and anti-CD28 (both functional grade-purified) monoclonal antibodies were bought from eBioscience (San Diego, CA). 3H metabolic activation solution was purchased from Moltox (Boone, NC). RPMI 1640 medium was purchased from HyClone (Logan, UT). Additional sources and vendors of specific reagents, such as antibodies and kits, are indicated as needed below.

### Animals

Since children are more susceptible to DEHP hazards, weanling mice were used in this study. Weanling BALB/c mice (both genders, 3–4 weeks of age, 12–16 g) were obtained from the Experimental Animal Center of Nantong University (Nantong, China). Mice were housed (gender separated) in a temperature-controlled (24–26°C) room at 60 ±5% humidity under a 12-h light/dark cycle. All mice had ad libitum access to OVA-free rodent chow and filtered distilled water. Mice were quarantined for at least 7 days before study initiation.

All experiments involving mice were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications, revised 2011) and were approved by the Chinese National Committee to the Use of Experimental Animals for Medical Purposes, Jiangsu Province. All efforts were made to minimize the number of mice used with regard for alleviation of suffering.

### In vivo exposure and immunization protocols

Since gastrointestinal exposure best reflects actual human exposures, a murine model of DEHP gavage with OVA sensitization was established here. In brief, BALB/c mice were randomly divided into eight groups (8 mice/group; four male and four female): four were comprised of DEHP (30, 300, 3000 μg/kg) or corn oil gavage with OVA sensitization and the other four were DEHP (30, 300, 3000 μg/kg) or corn oil gavage with normal saline [sham-sensitization]. The DEHP gavage dosages were chosen based on current risk assessment parameters and safety limit values.

For the exposure, DEHP was dissolved in laboratory-grade corn oil; the mice were then gavaged with DEHP or corn oil at equivalent volume once a day from Day 1 to Day 28 (28 times) of the study. For the mice that were to be sensitized with OVA, a solution of 100 μg OVA in 50 μl normal saline was injected into two footpads (double subcutaneous injection) on Day 7 and Day 16 of the study. Parallel DEHP-only and vehicle-only control groups were sham-sensitized with 50 μl normal saline on each of those days. On Day 29, all mice were euthanized by cervical dislocation and their spleens harvested at necropsy.

### Isolation of splenic CD4⁺ CXCR5⁺ Tfh cells

From each spleen, single cell suspensions of splenic lymphocytes were prepared by density gradient centrifugation (to help remove red blood cells, etc.) and the final purified cells were re-suspended in phosphate-buffered saline (PBS, pH 7.4) to 5 × 10⁶ cells/ml. Splenic CD4⁺ Tfh cells were then isolated from each preparation using a Mouse CD4⁺ T-Cell Isolation Kit II (Miltenyi, Bergisch Gladbach, Germany). Splenic CD4⁺ CXCR5⁺ Tfh cells were further purified from all other CD4⁺ cells present by magnetic separation using phycoerythrin (PE)-conjugated anti-CXCR5 and then anti-PE MicroBeads (Miltenyi) in succession, according to manufacturer protocols. The cells were then re-suspended in PBS and counted using a hemocytometer. The purity of isolated CD4⁺ CXCR5⁺ Tfh cells was subsequently seen to be >95% (flow cytometry). The isolated cells were all found to maintain good viability and be able to undergo proliferation.

### SLAMF1 and SAP mRNA expression in the isolated Tfh cells

Total RNA was extracted from aliquots of isolated splenic CD4⁺ CXCR5⁺ Tfh cells (10⁶) of each mouse in each group using...
Spleen lymphocytes from each group (10^6) were incubated for 20 min at 4 °C in the dark in a solution containing 2.5 µg/ml fluorescein isothiocyanate (FITC)-anti-mouse CD4, 2.5 µg/ml PE-anti-mouse CXCR5, and 5 µg/ml allophycocyanin (APC)-anti-mouse SLAMF1 (eBioscience). After centrifugation/washing with PBS, the cells were re-suspended in PBS and then underwent flow cytometric analysis in a FACS Calibur instrument (BD Bioscience, San Jose, CA) to assess SLAMF1 expression levels on the cells as a function of host treatment. All data were analyzed using FCS Express software (BD Bioscience). A minimum of 10,000 events/sample was acquired.

**SAP protein expression in T_{fh} cells**

Whole protein was extracted from each set of splenic CD4^+CXCR5^+T_{fh} cells ( aliquots of 10^6 cells) using M-PER mammalian protein extraction reagent (Pierce, Rockford, IL). Total protein concentration in each extract was measured using the NanoDrop One spectrophotometer. Aliquots of total protein (20 µg/lane) were then resolved over 15% gels (SDS-PAGE) and the separated proteins then electrotransferred onto poly-vinylidene-difluoride (PVDF) membranes (Millipore, Boston, MA). Two sets of membranes were prepared for each group. Each membrane was then blocked with 5% fat-free milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBS-T) for 2 h at room temperature (RT), and then incubated at RT for 2 h in a solution of TBS-T containing any one of the following primary rabbit monoclonal antibodies at 0.1 µg/ml: anti-mouse SAP or anti-mouse GAPDH (Abcam, Boston, MA). After rinsing with TBS-T buffer, each membrane was then treated with a solution of TBS-T containing secondary antibody (0.1 µg/ml horseradish peroxidase-conjugated goat anti-rabbit IgG antibody [Abcam]) and incubated at RT for 2 h. After gentle washes with TBS-T to remove unbound secondary antibody, all membranes underwent development with enhanced chemiluminescence reagents (Amersham, Little Chalfont, Buckinghamshire, UK). The intensity of relative protein expression was then measured in a chemiluminescence imaging system (Syngene, Cambridge, Cambridgeshire, UK) using Image J analysis software (National Institutes of Health, Bethesda, MD).

**Ex vivo analyses of effects of DEHP on T_{fh} cells**

For this study, additional sets of BALB/c mice were sensitized with OVA (100 µg in 50 µl normal saline) by double subcutaneous injection into the footpads on Day 1 and Day 10. At 10 days post-sensitization, all mice were euthanized by cervical dislocation and their spleens harvested at necropsy. T_{fh} cells from each mouse were then isolated as above.

For these in vitro studies, the T_{fh} cells were counted and diluted with complete medium (RPMI 1640 containing 10% fetal bovine serum [FBS, South America source], but without any antibiotics) to 5 x 10^5 cells/ml. The cells were then transferred into 6-well culture plates (2 ml suspension/well) and cultured at 37 °C in a 5% CO2/95% air humidified incubator. After 24 h, the T_{fh} cells were transiently transfected with Slamf1 siRNA (to silence Slamf1 gene) or with NC siRNA using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer protocols. The mRNA knockdown efficiency was evaluated 48 h post-transfection by qRT-PCR; protein knockdown efficiency was evaluated 72 h post-transfection by Western blot (see above for details of qRT-PCR and Western blot protocols).

Upon completion of the above, these T_{fh} cells were seeded into 24-well plates (10^6 cells/ml/well) and randomly delegated into one of six groups: (1) Control (non-transfected), (2) NC siRNA cells, (3) Slamf1 siRNA cells, (4) DEHP-treated control cells, (5) DEHP-treated NC siRNA cells, and (6) DEHP-treated Slamf1 siRNA cells. For the exposures, DEHP solution was dissolved in 0.1% DMSO and added to wells for groups (4), (5), and (6) at a final concentration of 50 µg/ml. Wells for cells in groups (1), (2), and (3) received only 0.1% DMSO at an equivalent volume. After this, 59 metabolic activation mixture (0.4 ml/well) was added to all the wells (specifically, to promote DEHP metabolism in group (4), (5), and (6) wells). Anti-mouse CD3 (100 µl, final level in well of 0.5 µg/ml) and anti-mouse CD28 (100 µl, final level in well of 0.5 µg/ml) antibody were then both added to each well to maintain induction and culture. Doses of all chemicals/antibodies above were chosen based on reference data/preliminary experimental results. The plates were then incubated at 37 °C for 72 h at which point cell culture supernatants were collected and frozen for later analysis. The cells were collected, washed, and prepared for analysis in the various protocols outlined below.

**SLAMF1, SAP, bcl-6 and c-MAF mRNA expression in ex vivo-treated T_{fh} cells**

Total RNA was extracted from the original Slamf1 siRNA- or NC siRNA-transfected cultures, as well as each Groups (1) – (6) T_{fh} culture above using Trizol. Thereafter, the isolated RNA was measured and evaluated in real-time qRT-PCR using the same protocols outlined above. Besides primers for Slamf1, Sh2d1a and Gapdh, another other primer pairs were used: Bcl-6 (F) 5'-CCTGAGGAAAGCAATATCA-3', (R) 5'-CGGTCTGTAGGGAACTCTTC-3'; c-Maf (F) 5'-GCAGAGACGCAGCTCGAGG TCG-3', (R) 5'-CGAGCTTTGGCCCTGAACTAGC-3'; and, Gapdh (F) 5'-GCTTGAAGGTGTGCTCCCTAG-3' and (R) 5'-AGAAGCCAGGTTCACCCAGAC-3'. As before, relative mRNA expression levels in each cell set were all normalized to the Gapdh reference and calculated using the 2^ΔΔ Ct method.
SLAMF1 and SAP protein expression in ex vivo-treated Tfh cells

Whole protein was extracted from the original Slamf1 siRNA- or NC siRNA-transfected cultures, as well as each Group (1)–(6) Tfh cultures above using M-PER mammalian protein extraction reagent. Once isolated and quantified, aliquots of the samples then underwent Western blot analyses as outlined above. In this case, three membranes were generated for each group to permit simultaneous analyses of three individual proteins. For these studies, the primary rabbit monoclonal antibodies used were anti-mouse-SLAMF1, -SAP, or -GAPDH.

Bcl-6 and c-MAF expression in ex vivo-treated Tfh cells

Aliquots containing Tfh cells (10⁶) from each of the culture groups were incubated for 20 min at RT in a solution of PBS containing 2.5 μg/ml of anti-mouse CD16/CD32 (eBioscience) to block nonspecific binding. After centrifugation, to permit surface staining, the cells were re-suspended in PBS containing 2.5 μg/ml each of FITC-anti-mouse CD4 and PE-anti-mouse CXCR5 (eBioscience). The cells were then incubated for 30 min at 4°C in the dark, and then washed three times with PBS. For intracellular staining, the cells were then fixed and permeabilized with Fixation/Permeabilization working solution using a Transcription Factor Staining Buffer Kit (eBioscience) according to manufacturer protocols. The cells were then treated with PBS containing 0.3 μg/ml APC-anti-mouse Bcl-6 or 1.25 μg/ml eFlour 660-anti-mouse c-MAF (eBioscience) for 30 min at 4°C in the dark, and then washed three times with kit-provided permeabilization buffer. The final post-stained cell pellets were re-suspended in 200 μl PBS, and flow cytometry analysis was performed in the FACS Calibur system. All data were analyzed using FCS Express software; minimum of 10,000 events/sample was acquired.

Quantification of secreted IL-21 and IL-4 from ex vivo-treated Tfh cells

The amounts of the Tfh cell characteristic cytokines IL-21 and IL-4 in the supernatants that were collected after 72 h of culturing of the cells in each treatment group were measured using a Mouse IL-21/IL-4 Platinum ELISA Kit (eBioscience), according to manufacturer instructions. Cytokine levels were determined using a spectrometer enzyme-labeled instrument (Bio-Rad, Hercules, CA); all values were extrapolated from standard curves generated from kit-provided mouse IL-21 and IL-4 standards examined in parallel. The lower limits of quantification for IL-21 and IL-4 were 5.0 and 2.0 pg/mL, respectively. All samples were analyzed in triplicate.

Results

Effects of DEHP exposure on OVA-sensitized weanling mice spleen Tfh cell surface receptor SLAMF1 expression

Real-time qRT-PCR and flow cytometric analyses were used to assess SLAMF1 mRNA/protein expression in murine spleen Tfh cells from each group. Relative to OVA sensitization groups, most of the measured values for cells from DEHP-only and vehicle-only control groups without OVA sensitization were quite small, indicating DEHP oral administration alone could not impart an allergen effect. In OVA-sensitized weanling mice, as a result of DEHP exposure, SLAMF1 mRNA expression and the percentage of CXCR5⁺SLAMF1⁺ Tfh cells among the entire CD4⁺ Tfh cell populations notably increased compared to corresponding values seen with the corn oil control mice (Figure 1(A,B), Table 1 in supplementary material). The effect was apparent across all the levels of DEHP tested (30, 300, 3000 μg/kg) although the effect itself was not dose-dependent.

Effects of DEHP exposure on OVA-sensitized weanling mice spleen Tfh cell adaptor protein SAP expression

Data from real-time qRT-PCR and Western blots of SAP in isolated Tfh cells showed the majority of values for SAP with cells from DEHP-only and vehicle-only control groups without OVA sensitization were very low, suggesting DEHP intake (orally) alone was not antigenic. In OVA-sensitized weanling mice, SAP mRNA and protein expression levels in isolated cells from mice that had received any of the tested levels of DEHP were significantly higher than in cells from the corn oil control (Figure 2(A,B), Table 2 in supplementary material). The effect was apparent across all DEHP levels tested (30, 300, 3000 μg/kg) though the effect itself was not dose-dependent.

Influence of Slamf1 siRNA transfection (with DEHP exposure) on Tfh cell SAP expression

As SLAMF1 has a pivotal role in signal transduction pathways (Sawada 2012; Hu et al. 2013; Zhong and Veillette 2013a, 2013b), an siRNA-targeting Slamf1 gene was used to silence Slamf1 expression in the Tfh cells. As seen in Figure 3(A), mRNA and protein expression levels of SLAMF1 in the Tfh cells was significantly reduced after transfection, proving knockdown was satisfactory. To explore the influence of DEHP on SLAMF1-SAP signaling pathway in Tfh cells when silencing Slamf1 gene, SAP mRNA/protein expression in Tfh cells from each group were evaluated by real-time qRT-PCR and Western blots. The data showed that SAP mRNA/protein expression levels from Tfh cells from the DEHP exposure (no silencing) group were higher than those of control group Tfh cells. After transfection with Slamf1 siRNA, SAP mRNA/protein expression levels decreased in the DEHP + Slamf1 siRNA group compared to that in cells from the DEHP exposure (no silencing) group or the DEHP + NC siRNA group (Figure 3(B,C), Table 3 in supplementary material).

Influence of Slamf1 siRNA transfection (with DEHP exposure) on Tfh cell nuclear transcription factor expression

Both Bcl-6 and c-MAF are regarded as intrinsic Tfh cell regulators (Kroenke et al. 2012; Liu et al. 2012; Andris et al. 2017). To clarify the influence of DEHP on Tfh cell transcriptional control, mRNA/protein expression levels of Bcl-6 and c-MAF

Statistical analysis

Results are expressed as means ± SD. Statistical differences between groups were evaluated using an analysis of variance (ANOVA). The criterion for significance in all cases was a p-value < 0.05. Linear correlation analysis was also performed. All data were analyzed using IBM SPSS Statistics 20.0 software (IBM Corp, Armonk, NY).
transcription factors were examined in T<sub>fh</sub> cells from each group – with and without Slamf1 gene silencing. Using real-time qRT-PCR and flow cytometry, Bcl-6 and c-MAF mRNA expression levels and the percentage of CXCR5<sup>+</sup>/BCL-6<sup>+</sup>/CXCR5<sup>+</sup>/c-MAF<sup>+</sup> T<sub>fh</sub> cells among all CD4<sup>+</sup> T cell populations were evaluated. The results indicated the values associated with the cells from the DEHP exposure (no silencing) group increased compared to those with the cells from the control group. After transfection, both Bcl-6 and c-MAF mRNA levels, as well as levels of CD4<sup>+</sup>/BCL-6<sup>+</sup>/CD4<sup>+</sup>/CXCR5<sup>+</sup>/c-MAF<sup>+</sup> T<sub>fh</sub> cells, decreased in the DEHP + Slamf1 siRNA cells relative to those in the DEHP exposure (no silencing) or DEHP + NC siRNA cells (Figure 4(A,B), Table 4 in supplementary material).

**Influence of Slamf1 siRNA transfection with DEHP exposure on T<sub>fh</sub> cell cytokine secretion**

Mature T<sub>fh</sub> cells can synthesize some cytokines, especially IL-21 and IL-4 (Luthje et al. 2012; Read et al. 2016; Sahoo et al. 2016). To monitor the influence of DEHP on T<sub>fh</sub> cell cytokine secretion when the Slamf1 gene was silenced, secretion of IL-21 and IL-4 by T<sub>fh</sub> cell cultures generated from each treatment group was evaluated by ELISA. The secreted amounts of IL-21 and IL-4 from the DEHP exposure (no silencing) group were higher than those from cells from control group T<sub>fh</sub> cells. As a result of Slamf1 siRNA transfection, relative to levels produced by T<sub>fh</sub> cells from the DEHP exposure (no silencing) group or the
Figure 3. Influence of Slamf1 siRNA transfection (with DEHP exposure) on Tfh cell adaptor protein SAP expression. (A) SLAMF1 protein (Western blot) and SLAMF1 mRNA expression (real-time qRT-PCR). (B) SAP protein expression (ratio to GAPDH; Western blot). (C) SAP mRNA expression (normalized to Gapdh; real-time qRT-PCR). Data shown are means ± SD, n = 6 samples/group. "p < 0.001 vs. control group or NC siRNA group; "p < 0.001 vs. control group; "p < 0.001 vs. DEHP exposure group or DEHP + NC siRNA group; ns = p > 0.05 vs. control group or NC siRNA group.

Figure 4. Influence of Slamf1 siRNA transfection (with DEHP exposure) on Tfh cell nuclear transcription factor expression. (A) Expression percentages of CXCR5+ BCL-6+ and CXCR5+ c-MAF+ Tfh cells gated on CD4+ T-cell populations (%) (flow cytometry). (B) CXCR5 and c-MAF mRNA (normalized to Gapdh; real-time qRT-PCR). Data shown are means ± SD, n = 6 samples/group. "p < 0.01, "p < 0.001 vs. control group; "p < 0.001 vs. DEHP exposure group or DEHP + NC siRNA group; ns = p > 0.05 vs. control group or NC siRNA group.
Recent epidemiology studies have uncovered a positive association between host exposure to DEHP and increased prevalence of allergic diseases in infants and juveniles (North et al. 2014; Beko et al. 2015; Li et al. 2017). One experiment between host exposure to DEHP and increased prevalence of allergic diseases in infants and juveniles (North et al. 2014; Beko et al. 2015; Li et al. 2017). One experiment showed that DEHP exposure-related anaphylactic responses could be ascribed to induction of Tfh cell hyper-function (Han et al. 2014). The Tfh cell, a newly confirmed CD4+ T cell subset, until recently was considered a key player in humoral immunity by assisting B-cells in their capacity to produce antibodies (Johnston et al. 2009; Nurieva et al. 2009). It has been subsequently shown these cells have an even larger role in host immunocompetence. Specifically, it has been shown that abnormal differentiation and hyperfunction of Tfh cells can cause an immune imbalance in a host (Ma and Deenick 2014; Ueno et al. 2015; Varricchi et al. 2016; Gensous et al. 2018).

As with many immune cells, Tfh cells may respond to stimulation through various receptors (Webb and Linterman 2017). As the prototypic member of SLAM family, SLAMF1 is a Type I transmembrane glycoprotein widely-expressed on the surface of T-cells. It is rapidly induced after naïve T-cell activation (de Calisto et al. 2014; Chen et al. 2017). SLAMF1 has been viewed as a distinct co-stimulatory molecule used to strengthen and sustain T-cell receptor (TCR) signals. As such, it is believed to play a role in the differentiation and maturation of Tfh cells (Cannons et al. 2011; Ma and Deenick 2011; Gordiienko et al. 2018). As such, it has been proposed that SLAMF1 receptors participate in modulating humoral immune responses, and that differences in subsequent cellular responses depend on the type and intensity of stimuli. In the present study, it was found that DEHP exposure (by oral intake) led to elevated SLAMF1 expression in splenic Tfh cells from OVA-sensitized weanling mice, implying that SLAMF1 receptor expression on the Tfh cell surface might be somehow related to any adjuvant effects/toxicities associated with DEHP exposure.

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Recent epidemiology studies have uncovered a positive association between host exposure to DEHP and increased prevalence of allergic diseases in infants and juveniles (North et al. 2014; Wang et al. 2014; Beko et al. 2015; Li et al. 2017). One experiment showed that DEHP exposure-related anaphylactic responses could be ascribed to induction of Tfh cell hyper-function (Han et al. 2014). The Tfh cell, a newly confirmed CD4+ T cell subset, until recently was considered a key player in humoral immunity by assisting B-cells in their capacity to produce antibodies (Johnston et al. 2009; Nurieva et al. 2009). It has been subsequently shown these cells have an even larger role in host immunocompetence. Specifically, it has been shown that abnormal differentiation and hyperfunction of Tfh cells can cause an immune imbalance in a host (Ma and Deenick 2014; Ueno et al. 2015; Varricchi et al. 2016; Gensous et al. 2018).

As with many immune cells, Tfh cells may respond to stimulation through various receptors (Webb and Linterman 2017). As the prototypic member of SLAM family, SLAMF1 is a Type I transmembrane glycoprotein widely-expressed on the surface of T-cells. It is rapidly induced after naïve T-cell activation (de Calisto et al. 2014; Chen et al. 2017). SLAMF1 has been viewed as a distinct co-stimulatory molecule used to strengthen and sustain T-cell receptor (TCR) signals that, in turn, allow Tfh cells to selectively provide help to B-cells (Cannons et al. 2011; Ma and Deenick 2011; Gordiienko et al. 2018). As such, it has been proposed that SLAMF1 receptors participate in modulating humoral immune responses, and that differences in subsequent cellular responses depend on the type and intensity of stimuli. In the present study, it was found that DEHP exposure (by oral intake) led to elevated SLAMF1 expression in splenic Tfh cells from OVA-sensitized weanling mice, implying that SLAMF1 receptor expression on the Tfh cell surface might be somehow related to any adjuvant effects/toxicities associated with DEHP exposure.

Its unique functional domain indicates that SLAMF1 is not only a surface receptor but also a signal transduction factor (Wu and Veillette 2016; Dragovich and Mor 2018). SLAMF1 carries one or more paired ITSM in its cytoplasmic tail; upon external stimulation, SLAMF1 can selectively bind SH2-containing intracellular adaptor molecules, chiefly SAP. SAP is a small adaptor protein that links SLAM family receptors to active signaling molecules like the Src family protein tyrosine kinase Fyn (Dong et al. 2012; Proust et al. 2012; Zhong and Veillette 2013a; Samanta and Mukherjee 2017). The present study revealed that exposure to DEHP (by oral intake) led to a boost in SAP adaptor expression inside Tfh cells from OVA-sensitized weanling mice; these changes paralleled those in expression of SLAMF1 receptor on the cells. In addition, ex vivo exposure to DEHP also acted in an adjuvant manner to reinforce SAP expression in Tfh cells.

The studies here also showed that transfection of the host Tfh cells with Slamf1 siRNA reduced the induced-expression of SAP caused by DEHP. These results suggested that a SLAMF1-SAP-dependent pathway might be involved in any DEHP-induced abnormal differentiation and/or hyperfunction of Tfh cells. It was interesting to note that there was no significant down-regulation of SAP expression after Slamf1 gene silencing in cells without any DEHP exposure. It is possible that SAP signaling transduction is controlled by multiple SLAM family members other than SLAMF1 under normal physiological conditions.

To further clarify whether the abnormal differentiation and hyperfunction of Tfh cells due to DEHP exposure was being mediated by SLAMF1 receptors, an ex vivo intervention model was used to investigate DEHP immunotoxicity in Tfh cells. In non-silenced Tfh cells from OVA-sensitized hosts, DEHP displayed an adjuvant effect in promoting expression of transcription factors (Bcl-6 and c-MAF) and cytokines (IL-21 and IL-4). Transfection of Tfh cells with Slamf1 siRNA attenuated these effects. Simply silencing Slamf1 gene without DEHP exposure had no influence on expression of these four proteins. These coordinated changes seen in the activated Tfh cells was not unexpected. It is known that Bcl-6 can induce substantial expression of surface markers and c-MAF can induce synthesis of IL-21 and IL-4 (Kroenke et al. 2012; Liu et al. 2012; Andris et al. 2017). The observed changes in relative expression of Tfh cells among all the splenic Tfh cells was also likely linked to the above-noted changes in Bcl-6 expression. There is evidence to show the Tfh cell differentiation program is closely dependent upon interactions among certain cooperative and antagonistic transcription factors (Lu et al. 2011; Cannons et al. 2013). Thus, when taken together, the data from the current study indicate that SLAMF1 might be the specific surface receptor for DEHP (and/or its metabolites) during any targeting of host Tfh cells, with any induced changes leading to abnormal differentiation and/or hyper-function.

Conclusions

The present study demonstrated that the environmental pollutant DEHP, via a SLAMF1-mediated pathway, could interfere with Tfh cell differentiation and cytokine secretion. The findings here provide a new theoretical basis to postulate novel mechanisms underlying DEHP immunotoxicity. Research building on these findings could eventually contribute to improving the prevention and/or treatment of DEHP-related allergic diseases.
Disclosure statement
No potential conflict of interest was reported by the authors.

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