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Unexpected heterogeneity of multifunctional T cells in response to superantigen stimulation in humans

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Abstract

Toxic shock syndrome (TSS) is a potentially life threatening condition characterized by fever, rash, shock, and multi-organ failure. Staphylococcal enterotoxin B (SEB) is a well characterized superantigen that has been shown to play an important role in TSS. Although the precise mechanisms by which SEB and other superantigens cause TSS are unknown, induction of a pro-inflammatory cytokine cascade appears central to this phenomenon. We show that CD4⁺ and CD8⁺ T effector/memory (T_{EM}) and other subsets produce IL-17A following SEB stimulation. We also show that IL-17A is co-produced with other pro-inflammatory cytokines (i.e., IL-2, IFN- γ and TNF- α). These responses are significantly different than those elicited by mitogenic stimulation. Multifunctional IL-17A producing cells possess markers typical of the T_H17/T_C17 and T_H1 subsets, including CCR6, IL-22, and transcription factors retinoic acid receptor-related orphan nuclear receptor (ROR)- γ t and T-bet. These results suggest a possible role for IL-17A-producing multifunctional T cells in the pathogenesis of TSS.

Keywords

IL-17A; multifunctional T cells; superantigen; T_H17; Staphylococcal enterotoxin B

1. Introduction

Toxic shock syndrome (TSS) is a severe, toxin-mediated disease that is caused by superantigen producing strains of the Gram-positive cocci *Staphylococcus aureus* and *Streptococcus pyogenes* [1]. Superantigens are able to bypass the need for processing by antigen presenting cells. They complex directly with most major histocompatibility complex (MHC) II molecules and then bind to the conserved T cell receptor- β (TCR- β) subunit encoded by specific V β gene segments outside of the antigen binding groove [2]. In addition, binding of the CD28 homodimer interface is required for the induction of cytokine production [3]. In this manner, T cell recognition of a superantigen is independent of clonal specificity and superantigens typically interact with up to 20% of the peripheral T cells [2–6]. Staphylococcal enterotoxin B (SEB) is a well characterized superantigen produced by

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toxigenic strains of *Staphylococcus aureus* [4, 7], which has been implicated in non-menstrual TSS [8–10]. The precise mechanism by which SEB induces TSS is not known; however, there is evidence that production of pro-inflammatory cytokines plays an important role in pathogenesis [7]. SEB stimulation of T cells results in release of many cytokines including tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-2, IL-4, IL-6, and interferon (IFN)- γ [5].

Recently, IL-17A has been shown to be a mediator of neutrophil stimulation and mobilization [11]. *In vitro*, IL-17A works synergistically with TNF- α and IFN- γ resulting in increased production of multiple chemokines [11]. Elevated plasma levels of IL-17A have been detected in a mouse model of sepsis, and it is hypothesized to contribute to the cytokine storm by causing increased production of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 [12]. Additionally, studies in a mouse model of TSS showed elevated serum levels of IL-17 in response to systemic administration of SEB [13]. IL-17A is known to be produced by multiple cell types including CD4+ and CD8+ T cells, as well as $\gamma\delta$ T cells [11, 14–17]. CD4+ T helper (T_H) 17 cells are thought to be the major producers of IL-17A. The T_H17 subset is distinct from T_H1 and T_H2 subsets and is characterized by the production of IL-17A as well as expression of the retinoic acid receptor-related orphan nuclear receptor (ROR)- γ t transcription factor and the CC-chemokine receptor (CCR) 6 [14, 18]. Other cytokines including IL-17F and IL-22 are also produced by T_H17 cells [11, 14].

To determine whether or not IL-17A is produced in response to SEB stimulation in humans and to characterize IL-17A producing cells, multichromatic flow cytometry was performed. Panels with 12–13 colors were developed and used to allow the simultaneous detection of multiple intracellular cytokines/chemokines, markers of cytolytic capacity, and expression of transcription factors and homing molecules. Here we describe the results of these studies which contribute additional insights into the complex mechanisms underlying TSS pathogenesis.

1. Materials and Methods

2.1 Volunteers and isolation of peripheral blood mononuclear cells (PBMC)

PBMC collected from 26 healthy adult volunteers, recruited from the Baltimore-Washington area and University of Maryland at Baltimore campus, were used in this study. Informed consent was obtained and all procedures were approved by the University of Maryland at Baltimore IRB. PBMC were isolated immediately after blood draws by density gradient centrifugation and cryopreserved in liquid nitrogen following standard techniques [19, 20].

2.2 Ex vivo stimulation

PBMC were thawed and rested overnight at 37 °C. Cells were then resuspended in RPMI 1640 media (Gibco, Carlsbad, CA) supplemented with 100 U/mL penicillin (Sigma, St. Louis, MO), 100 μ g/mL streptomycin (Sigma), 50 μ g/mL gentamicin (Gibco), 2mM L-glutamine (Gibco), 2.5mM sodium pyruvate (Gibco), 10mM HEPES buffer (Gibco), and 10% fetal bovine serum (Gemini Bioproducts, West Sacramento, CA) at a concentration of 1×10^6 cells/mL in 6 or 12 well plates. SEB (Sigma) was added at a concentration of 10 μ g/mL and cells were incubated at 37°C in 5% CO₂. After 2 hours, Golgi Stop (containing monensin) and Golgi Plug (containing brefeldin A) from BD were added at concentrations of 0.5 μ l/mL and cultures continued overnight at 37°C in 5% CO₂. Media alone was used as a negative control. Phorbol myristate acetate (PMA) (Sigma; 50ng/mL) and ionomycin (Sigma; 500 ng/mL) were used as positive controls for stimulation of T_H17/Tc17 cells. Golgi Stop (0.7 μ l/mL) and Golgi Plug (1 μ l/mL) were added one hour after PMA and ionomycin and cultures continued for 3 additional hours. In studies using phytohemagglutinin (PHA), stimulation was performed with 2 μ g/mL PHA followed by

incubation at 37°C in 5% CO₂ for 2 hours and addition of Golgi Stop (containing monensin) and Golgi Plug (containing brefeldin A) from BD prior to overnight incubation as described for SEB stimulation.

2.3 Intracellular staining of cytokines and flow cytometric analyses

Following stimulation as described above, cells were plated in 96-well V-bottom plates for staining. Cells were washed once with staining buffer (phosphate buffered saline with 0.5% BSA and 0.1% sodium azide) and stained for live/dead discrimination using Invitrogen LIVE/DEAD fixable violet or yellow dead cell stain kit (Invitrogen, Carlsbad, CA). Fc receptor blocking was performed with human immunoglobulin (Sigma; 3 µg/mL) followed by surface staining, performed as previously described [21]. Briefly, cells were stained with CD14-Pacific Blue or Brilliant violet (BV) 570 (TuK4, Invitrogen or M5E2, Biolegend, San Diego, CA) and CD19-Pacific Blue or BV570 (SJ25-C1, Invitrogen or HIB19, Biolegend), CD3-biotin or BV650 (UCHT1, Beckman Coulter, Danvers, MA or OKT3, Biolegend), CD4-Qdot 655 or PE-Cy5 (S3.5, Invitrogen or RPA-T4, BD), CD8-Qdot 705 or PerCP-Cy5.5 (3B8, Invitrogen or SK1, BD), CD45RA-Qdot 605 or biotin (MEM56, Invitrogen or HI100, BD), CD62L-PE-Cy5 or APC-EF780 (Dreg 56, BD, Franklin Lakes, NJ or eBioscience), CCR6-biotin (11A9), and/or CD107a/b (eBioH4A3/eBioH4B4, eBioscience, San Diego, CA) at 4 °C for 30 minutes. The antibodies to CD107a and CD107b, directed to LAMP-1 and LAMP-2, respectively, were used to measure degranulation [22]. Staining with streptavidin-Pacific Orange or Qdot 800 (Invitrogen) was performed for panels that included biotin-conjugated monoclonal antibodies for 30 minutes at 4°C. The cells were then fixed and permeabilized using IC fixation and permeabilization buffers from eBiosciences according to manufacturer's recommendations. Intracellular staining with IL-17A-PerCP-Cy5.5 or BV421 (eBio64DEC17, eBioscience or BL168, Biolegend), IL-2-PE-Cy7 or BV605 (MQ1-17H12, BD or Biolegend), IFN-γ-Alexa 647, PE-Cy7, or APC-Alexa 750 (B27, BD, or Invitrogen), TNF-α-Alexa 700 (MAb11, BD), IL-10-FITC (127107, R&D, Minneapolis, MN), MIP-1β-APC or PE (24006, R&D), IL-22-FITC (142928, R&D), RORγt-PE (AFKJS-9, eBioscience), T-bet-PerCP-Cy5.5 (O4-46, BD), and/or CD69-PE-Cy5, ECD, or PE (TP1.55.3, Coulter or FN50, eBioscience) was performed at 4 °C overnight. After staining, cells were fixed in 1% paraformaldehyde and stored at 4 °C until analyzed. Flow cytometry was performed using a customized LSRII flow cytometer (BD). Flow cytometry data were analyzed using WinList version 7 (Verity Software House, Topsham, ME), FLOCK (ImmPort, NIH web site www.immport.org), and SPICE version 5.1 (<http://exon.niaid.nih.gov>) software packages [23]. Graphs were generated using GraphPad Prism version 5.03 (Graphpad Software, San Diego, CA) and SPICE version 5.1 (<http://exon.niaid.nih.gov>).

2.4 Statistical analyses

All tests were performed using GraphPad Prism version 5.03 (Graphpad Software) and SPICE version 5.1 [23]. In flow cytometry experiments a response was considered significant if the differential in the number of positive events between experimental (SEB) and negative control (media) cultures was significantly increased by Chi-square tests. For SPICE analyses, a threshold value (below which all values are considered 0) of 0.3% positive cells was set based on the distribution of negative net values (SEB – media) as previously described [23]. P values < 0.05 were considered significant. Using SPICE, comparison of distributions was performed using a Student's T test and a partial permutation test as described [23].

3. Results

3.1 IL-17A is produced by CD4⁺ and CD8⁺ T_{EM} cells in response to SEB stimulation

PBMC from healthy volunteers were stimulated with SEB as described followed by intracellular cytokine staining and multichromatic flow cytometry. T_H (CD3⁺ CD4⁺ CD8[−]) and T_C (CD3⁺, CD8⁺ CD4[−]) cells were defined as naïve T cells (T_N; CD62L⁺ CD45RA⁺), T central memory (T_{CM}; CD62L⁺ CD45RA[−]), T effector memory (T_{EM}; CD62L[−] CD45RA[−]) and T effector memory CD45RA⁺ (T_{EMRA}; CD62L[−] CD45RA⁺) as previously described (Supplemental Figure 1 A). Following fixation and permeabilization, intracellular cytokine staining for IL-17A, IL-2, IFN- γ , TNF- α , IL-10, and MIP-1 β was performed to determine if IL-17A is produced in conjunction with other cytokines/chemokines. MIP-1 β staining was not performed in two volunteers due to temporary unavailability of the antibody. After staining, samples were analyzed by flow cytometry. The gating strategy is depicted in Supplemental Figure 1 A&B.

IL-17A was detected in CD4⁺ and CD8⁺ T cells (Figure 1A&B). All 18 volunteers tested showed production of IL-17A by CD4⁺ T cells and 14 of 18 volunteers showed production of IL-17A by CD8⁺ T cells. IL-17A was produced predominantly by the CD4⁺ T_{EM} subset in all volunteers with lower levels produced by the CD4⁺ T_{EMRA} subset in some volunteers. The median percentage of CD4⁺ IL-17A⁺ T_{EM} was 2.06% with a range from 0.27–13.48%. Levels of IL-17A produced by CD4⁺ T_{EMRA} were lower with a median of 0.51% and a range from 0–1.69%. As seen with CD4⁺ T cells, CD8⁺ T_{EM} were the predominant source of IL-17A with a median of 0.49% and a range from 0–4.04%. In only 7 of 18 volunteers were IL-17A⁺ cells identified in the CD8⁺ T_{EMRA} population, and these were at much lower levels (range 0–0.44%). One volunteer showed production of IL-17A by CD8⁺ T_{CM} cells (Figure 1B).

3.2 IL-17A is produced by multifunctional CD4⁺ and CD8⁺ T cells that co-produce IL-2, IFN- γ , and/or TNF- α , but not IL-10 or MIP-1 β

Because the induction of multifunctional T_H17 and T_C17 cells following TCR stimulation with SEB has not been reported in humans, we then investigated whether direct TCR stimulation of PBMC isolated from healthy subjects with SEB elicited multifunctional CD4⁺ and CD8⁺ T cells, and which, if any, pro-inflammatory cytokines were produced in conjunction with IL-17A. We observed that multifunctional CD4⁺ and CD8⁺ T cells were elicited in response to SEB stimulation. All possible combinations (64 total combinations) of 6 cytokines/chemokines (IL-17A, IL-2, IFN- γ , TNF- α , IL-10, and MIP-1 β) were analyzed using the Winlist FCOM function (Figure 1C–F and Figure 2).

In CD4⁺ T cells, TNF- α single positive cells and TNF- α and IL-2 double positive cells were the most prevalent responses (Figure 1C&D). Triple positive cells producing IL-2, IFN- γ , and TNF- α or those producing IL-17A, IL-2 and TNF- α were also identified, followed by quadruple positive cells producing IL-17A, IL-2, IFN- γ and TNF- α , single positive cells producing IL-2, and double positive cells producing IFN- γ and TNF- α . Other combinations were also identified at lower levels. Due to space constraints it was not feasible to show all 64 possible combinations generated by simultaneous measurement of 6 cytokines/chemokines; therefore, only the top 10 populations are shown in Figure 1C&D. IL-17A was found almost exclusively in multifunctional T cells in all volunteers. In CD4⁺ T cells, triple positive cells (producing IL-17A, IL-2, and TNF- α) and quadruple positive cells (producing IL-17A, IL-2, IFN- γ , and TNF- α) were the predominant IL-17A producing populations in all volunteers (Figure 1E&F). Lower levels of double positive cells producing IL-17A and TNF- α were also identified. Other IL-17A⁺ populations were sometimes present at low levels; however, heterogeneity among volunteers was noted. Those IL-17A⁺

combinations with at least one value > 0.05% positive cells are shown in Figure 1E&F. CD4⁺ T_{EMRA} cells had lower levels of IL-17A production than CD4⁺ T_{EM}; however, IL-17A was still produced almost exclusively from multifunctional cells co-producing IL-2, IFN- γ , and TNF- α or IL-2 and TNF- α (data not shown).

CD8⁺ T_{EM} responses were also dominated by TNF- α single positive cells (Figure 2A&B). However, high percentages of multifunctional CD8⁺ T cells producing combinations of IL-2, IFN- γ , and TNF- α were also found. In the 5 volunteers in whom MIP-1 β production was measured, single positive cells producing MIP-1 β as well as multifunctional cells producing MIP-1 β , IL-2, IFN- γ , and/or TNF- α were also identified (Figure 2B). Although at lower levels, IL-17A was produced by CD8⁺ T_{EM} cells in 5 of 7 volunteers (Figure 2C&D). In those volunteers with a CD8⁺ T_{EM} IL-17A response, the IL-17A was produced by multifunctional cells co-producing IL-2, IFN- γ and TNF- α or IL-2 and TNF- α . As demonstrated for CD4⁺ T cells, other IL-17A⁺ populations were sometimes present at low levels (all combinations for which at least one volunteer showed >0.05% positive cells are shown in Figure 2C&D). CD8⁺ T cells showed extreme variability among volunteers accounting for many values of 0 (Figure 2C&D). There was no co-production of IL-17A with IL-10 by either CD4⁺ or CD8⁺ T cells.

3.3 Multifunctional IL-17A⁺ T cells do not co-express CD107a/b

In order to further investigate the presence of multifunctional IL-17A⁺ T cells and characterize them with regard to their cytolytic potential, PBMC from 11 healthy adult volunteers were rested overnight and stimulated with SEB as described above. Simultaneous staining for T cell memory subsets as well as 4 cytokines (IL-17A, IL-2, IFN- γ and TNF- α), and CD107a/b (widely accepted as a surrogate of cytolytic activity) was performed. These experiments confirmed that both CD4⁺ and CD8⁺ T_{EM} cells produced multiple cytokines simultaneously, including various combinations of IL-2, IFN- γ and TNF- α , with or without expression of CD107a/b (Figure 3A&B). In most volunteers, CD107a/b expression was higher in CD8⁺ T_{EM} cells than CD4⁺ T_{EM} cells. IL-17A was produced almost exclusively by CD4⁺ T_{EM} cells (Figure 3C). As described above (Figure 1), in CD4⁺ T_{EM} cells the predominant IL-17A⁺ populations co-produced IL-2 and TNF- α (triple positive) or IL-2, IFN- γ and TNF- α (quadruple positive). In only 2 volunteers, production of IL-17A by CD4⁺ cells was detected in combination with CD107a/b as well as IL-2, IFN- γ and TNF- α (Figure 3). As described in Figure 2, not all volunteers demonstrated production of IL-17A by CD8⁺ T_{EM} cells. In these responders, production of IL-17A was found to be dominant in multifunctional CD8⁺ T_{EM} cells that co-produced IL-2, IFN- γ and TNF- α (data not shown).

3.4 Not all multifunctional IL-17A producing CD4⁺ and CD8⁺ T cells co-express other markers of T_H17/T_C17 subsets

In order to determine if the IL-17A⁺ multifunctional T cells were T_H17/T_C17 cells that express ROR- γ t and produce IL-22 (markers of the T_H17/T_C17 subset), we stained PBMC from 5 volunteers with CD4, CD8, CD69, IL-17A, IL-2, IFN- γ , TNF- α , IL-22 and ROR- γ t. IL-22 was found to be produced by CD4⁺ CD69⁺ IL-17A⁺ IL-2⁺ IFN- γ ⁺ TNF- α ⁺ (quadruple positive) cells (Figure 4A and Supplemental Figure 3). Among quadruple positive CD4⁺ T cells, IL-22 production ranged from 1.87–10.45% with a median of 8.83%. These data were analyzed using WinList FCOM feature determining all possible combinations of the 5 cytokines and the ROR- γ t transcription factor examined. Traditional 2-parameter histograms and Boolean gating of CD4⁺ IL-17A⁺ and IL-17A⁻ populations is shown for a representative volunteer in Supplemental Figures 2 & 3. ROR- γ t expression was also found in a subset of the quadruple positive CD4⁺ T cells with a range from 0–4.67% (median 1.83%). Quadruple positive CD4⁺ T cells producing IL-22 and expressing ROR- γ t were identified in only 2 volunteers (0.32 and 0.93%). CD8⁺ CD69⁺ IL-17A⁺

IL-2+ IFN- γ + TNF- α + (quadruple positive) cells were present in lower numbers than CD4+ quadruple positive cells; however, 3 volunteers showed co-production of IL-22 (Figure 4B). There was a wide variation in the percentage of CD8+ quadruple positive cells that co-produced IL-22 (0.95–17.24%). No ROR- γ t expression was identified in the CD8+ quadruple positive cells. In 2 volunteers, the expression of CD161 (another marker associated with the T_H17/T_C17 subset) was determined. CD161 expression was found to be much higher in quadruple positive CD4+ T cells (i.e., IL-17A+ IL-2+ IFN- γ + TNF- α +; 45.11–73.36%) than triple positive cells (i.e., IL-17A+ IL-2+ TNF- α +; 9.83–19.52%) and IL-17A single positive cells (6.53–8.92%) (Figure 5). A similar trend was observed in CD8+ T cells (data not shown). Additionally, 3 volunteers were analyzed for co-expression of CCR6 (a homing molecule associated with the T_H17/T_C17 subset) by multifunctional IL-17A+ CD4+ T cells. Of quadruple positive (IL-17A+ IL-2+ IFN- γ + TNF- α +) CD3+ CD4+ CD69+ T cells 42.9–60.7% co-expressed CCR6 following stimulation with SEB (Figure 6).

3.5 Expression of T_H17/T_C17 and T_H1/T_C1 transcription factors in multifunctional IL-17A+ T cells in response to SEB stimulation

Not all multifunctional IL-17A+ T cells co-express markers of T_H17/T_C17 subset, particularly transcription factor ROR- γ t. We therefore investigated the expression of transcription factors ROR- γ t (T_H17/T_C17) and T-bet (T_H1/T_C1) to determine if these multifunctional cells were more consistent with T_H1/T_C1 subsets. We identified 7.14–9.84% of CD4+ quadruple positive (IL-17A+ IL-2+ IFN- γ + TNF- α +) T cells co-expressing ROR- γ t. However, we found considerably higher expression for T-bet, ranging from 78.95–90% of CD4+ quadruple positive cells (IL-17A+ IL-2+ IFN- γ + TNF- α +) (Figure 6). Due to the low quantities of CD8+ quadruple positive cells, these analyses were not deemed reliable in CD8+ cells.

3.6 IL-17A production after direct stimulation of the TCR by SEB differs from mitogenic stimulation

In order to determine if multifunctional IL-17A+ T cells were also present in response to other stimuli, PBMC from 6 healthy volunteers were stimulated with SEB, PHA, and PMA/ionomycin (non-TCR-dependent) followed by flow cytometric analyses to measure the simultaneous production of IL-17A, IL-2, IFN- γ , TNF- α , and MIP-1 β . IL-17A was produced by CD4+ T cells in response to all stimuli in all volunteers. Consistent with previous experiments, CD8+ T cells from only a subset of volunteers produced IL-17A in response to stimulation. As seen in response to SEB, the highest percentage of IL-17A+ cells was found in the T_{EM} population and this population was therefore used for subsequent analyses. The percentages of IL-17A+ CD4+ T_{EM} cells were significantly higher following PMA/ionomycin stimulation than for either SEB or PHA ($p < 0.05$ by Student's *t*-test). For those volunteers in whom a CD8+ T_{EM} response was detected, stimulation with PMA/ionomycin resulted in significantly higher production of IL-17A than stimulation with SEB ($p < 0.05$). There was no significant difference between IL-17A+ CD8+ T_{EM} responses to PMA/ionomycin versus PHA stimulation ($p = 0.687$). Moreover, no significant differences were observed in IL-17A production between SEB and PHA stimulation for either CD4+ T_{EM} or CD8+ T_{EM}. Multifunctional CD4+ T_{EM} IL-17A+ cells were identified in response to all 3 stimuli; however, the predominant populations varied by stimulant (Supplemental Figure 4 A&B). Triple positive cells producing IL-17A, IL-2, and TNF- α + were the predominant population stimulated by SEB, followed by IL-17A+ TNF- α + cells and IL-17A+ IL-2+ IFN- γ + TNF- α + cells (Supplemental Figure 4 A&B). These results were consistent with previous experiments (Figure 1). Interestingly, IL-17A-single positive CD4+ T cells were identified in response to both PHA and PMA/ionomycin, but not SEB (Supplemental Figure 4 A&B). Following PHA stimulation, the predominant CD4+ IL-17A+ populations

were single positive (IL-17A+), double positive (IL-17A+ TNF- α +) and triple positive (IL-17A+ IL-2+ TNF- α +) populations. Pie graphs displaying the proportions of IL-17A+ populations elicited by the different stimulations are also shown in Supplemental Figure 4A. Pie graphs were compared using a partial permutation analysis by SPICE as previously described [23]. There was a significant difference between PHA stimulation and SEB or PMA/ionomycin stimulation ($p < 0.01$) (Supplemental Figure 4A).

3.7 Unsupervised analysis using flow cytometry clustering without K (FLOCK) identifies additional multifunctional IL-17A producing CD4+ T_{EM} subsets

Given the complexity and heterogeneity observed in the concomitant production of multiple cytokines and expression of CD107a/b, we used FLOCK, a novel, unsupervised analysis program that uses computational methods to determine the number of unique populations in multidimensional flow cytometry data [24]. In order to further characterize the multifunctionality of IL-17A+ CD4+ T_{EM}, the same data that were analyzed for production of IL-17A, IL-2, IFN- γ and TNF- α and expression of CD107a/b by conventional, user guided methods (Supplemental Figure 1 A&B and Figure 3) were analyzed by FLOCK. Prior to FLOCK analyses, gating was performed as described in Supplemental Figure 1A to select CD3+ CD4+ T_{EM} events. Data for 11 volunteers were uploaded to the ImmPort website and FLOCK analyses performed. The number of unique populations identified varied among the volunteers (17–29 individual populations depending on the volunteer). In order to compare data from different volunteers, a cross-sample analysis was performed. In this cross-sample analysis, the populations identified in a single volunteer were applied to all samples. Seventeen unique CD69+ (recently activated) populations were identified in SEB stimulated samples compared to media controls (Table 1 and Figure 7). Population 14 (dark red) was IL-17A^{bright} (IL-17A^{br}) and present in all volunteers (Table 1, Figure 7, and Figure 8). An additional dim IL-17A population (IL-17A^{dim}, population 12, gold) was also observed. Population 14 had a higher MFI for IL-17A (392) than population 12 (240). As shown in Figures 7 and 8, population 14 was composed of quadruple positive cells, producing IL-17A, IL-2, IFN- γ and TNF- α but not expressing CD107a/b. In contrast, population 12 was composed of cells co-producing only IL-2 and TNF- α . In agreement with the traditional “guided” flow cytometric analyses described in Figures 1 to 3, IL-17A single positive populations were not identified by FLOCK. Table 1 shows the net percentage (SEB-media) of the 17 CD69+ populations (described in Figure 7) for each of the 11 volunteers. The percentages of quadruple positive cells (population 14) were highly variable among volunteers with ranges of 0.14–1.88% (Table 1). This is also in agreement with the variability seen in the percentages of quadruple positive cells identified by FCOM, 0.06–8.58% (Figure 3). There were, however, some differences in the ranges obtained by these 2 different analytical methods. As was the case using conventional analyses, IL-2+ (population 13) and TNF- α + (population 8) single producing cells were the predominant CD4+ CD69+ T_{EM} populations (mean 7.36 ± 2.22 and 6.25 ± 1.88 respectively) (Figure 7 and Table 1). The cytokine production profiles of the CD69+ populations along with the median values and interquartile ranges for the populations producing the measured cytokines are shown in Figure 7 (the details for each individual volunteer are shown in Table 1). Interestingly, 7 of the CD69+ populations identified by FLOCK (Populations 1, 2, 4, 6, 9, 11, and 16) did not produce any of the cytokines measured. These populations were separated primarily based on the differing intensities of CD69 and very dim cytokine staining, the intensity of which was below the cutoff for positivity defined in the guided analysis by staining with isotype and “fluorescence- minus-one” (FMO) controls. It is unclear whether these very dim populations are “true” very-low-level cytokine-producing cells because their level of staining was near the limit of sensitivity of the flow cytometer to separate signal from noise. Additionally, populations 5, 7, and 15 were all IL-2^{br} TNF- α ^d

and were separated by FLOCK as different populations based on relatively small differences in MFI of CD69 and other markers (Figure 7). The significance of these findings is unclear.

4. Discussion

In this manuscript we report that IL-17A is produced primarily by CD4⁺ and CD8⁺ T_{EM} T cell subsets, with some contribution by T_{EMRA} cells, in response to SEB stimulation. Although CD4⁺ cells are generally thought to be the predominant source of IL-17A, IL-17A production by CD8⁺ T cells has been shown in response to non-specific mitogen stimulation with PMA/ionomycin in animal models, as well as in human studies [16, 17, 25, 26]. Here we demonstrate that CD8⁺ T cells can also produce IL-17A in response to direct TCR stimulation, albeit at lower levels than those observed in CD4⁺ T cells.

Multifunctional T cells, those producing 2 or more cytokines simultaneously, have been shown to produce higher levels of individual cytokines, have enhanced function, and are more likely to correlate with protection from disease, when compared to single cytokine producing cells [27–29]. T cells co-producing IL-17A with IFN- γ or TNF- α , in response to PMA/ionomycin stimulation have been identified [30, 31]. Although previous studies have shown the co-production of IL-17A with other pro-inflammatory cytokines (*e.g.*, IL-2, IFN- γ , and/or TNF- α) [26, 30, 31], to our knowledge, this is the first study to examine the simultaneous production of IL-17A with other cytokines (IL-2, IFN- γ , TNF- α , and IL-22), chemokine receptors (CCR6), and transcription factors (ROR- γ t and T-bet) which allow the characterization of the T_H17/T_C17 and T_H1/T_C1-like properties of these cells during inflammatory processes in humans. Multifunctional IL-17A producing CD8⁺ T cells have been identified in response to *Salmonella* Typhi immunization, but the expression of homing markers and transcription factors associated with T_H17/T_C17 or T_H1/T_C1 was not addressed [26]. Unlike SEB, PMA/ionomycin stimulates and activates T cells non-specifically through activation of protein kinase C (PKC) and increase in concentrations of intracellular calcium without involvement of the TCR. The results described herein indicate that multifunctional IL-17A, IL-2, IFN- γ and TNF- α producing cells are elicited following direct TCR stimulation. Although the significance of these multifunctional IL-17A producing cells in TSS is at present unclear, it has been shown that multifunctional cells produce higher quantities of cytokines and are likely to be more effective than single cytokine producing cells [32]. Consensus is emerging that the quality of the T cell response is the most important factor in determining protection or undesired inflammatory responses against infectious organisms. In fact, it has been proposed that characterization of the effector/memory phenotype in conjunction with the multifunctional capabilities of T cells may be the best indicator of the quality of the response [32]. In addition to the “classical” inflammatory cytokines IFN- γ , TNF- α and IL-2, we also investigated MIP-1 β , also known as CC chemokine ligand 4 (CCL4), which is produced by many cell types and is involved in the recruitment of CD8⁺ T cells, neutrophils, and monocytes, and can therefore play an important role in inflammation. It has also been shown that SEB can induce the production of MIP-1 β by PBMC [33]. Interestingly, while MIP-1 β was produced by multifunctional CD8⁺ T cells, it was not produced in conjunction with IL-17A. Given the pro-inflammatory nature of the cytokines produced by IL-17A producing cells (IL-17A, IL-2, IFN- γ and TNF- α), it is likely that these multifunctional cells play a central role in eliciting and/or sustaining the cytokine storm associated with TSS.

The experiments investigating the co-production of IL-17A, IL-2, IFN- γ , and TNF- α in conjunction with the expression of CD107a/b confirmed the multi-functional nature of IL-17A producing human T cells in response to TCR stimulation by SEB. Antibodies to CD107a/b recognize LAMP-1 and LAMP-2, present in the membranes of cytotoxic granules, and can be detected on the cell surface following degranulation [22]. This

phenomenon has been observed predominantly, but not exclusively, in CD8⁺ T cells [32]. We observed that CD8⁺ IL-17A-producing cells do not generally possess cytolytic activity, suggesting that cytolytic activity by IL-17A⁺ cells is unlikely to be a major contributor to the pathology observed in TSS. These results are in agreement with studies in non-human primates showing that the majority of T_C17 cells do not co-produce granzyme [16]. This is, to our knowledge, the first study to simultaneously explore the cytolytic capacity and IL-17A production in CD4⁺ and CD8⁺ T cells isolated from healthy volunteers.

The multifunctional IL-17A producing T cells identified in these studies do not consistently express markers which have been typically associated with the T_H17/T_C17 cell subset, but the majority express the T_H1/T_C1 transcription factor T-bet. IL-22 is an important pro-inflammatory cytokine produced by T_H17 cells which has been detected in many chronic inflammatory conditions including psoriasis and multiple sclerosis and might therefore play a role in TSS [34, 35]. Interestingly, only a subset of CD4⁺ and CD8⁺ IL-17A-producing cells (*i.e.*, quadruple positive for IL-17A, IL-2, IFN- γ and TNF- α) co-produced IL-22, and only a subset of these cells co-expressed ROR- γ t (Figure 4).

In human PBMC, PMA/ionomycin stimulation induces a unique population of CD8⁺ T cells that possess markers typically associated with the T_H17 subset, production of IL-17A and IL-22, expression of ROR- γ t, IL-23R, and CCR6. This subset of CD8⁺ T cells, termed T_C17 cells, was identified in normal volunteers [25]. In addition to production of IL-17A, IL-22 and IFN- γ production were also measured and a predominance of multifunctional cells co-producing IL-17A, IL-22, and/or IFN- γ was noted in PBMC [25]. Multifunctional T_C17 cells were also identified in response to PMA/ionomycin in non-human primates and found to be depleted in simian immunodeficiency virus infection [16]. Here we show that the T_C17 subset can also be found in response to direct TCR stimulation with SEB; however, T_C17 cells do not appear to be the predominant source of IL-17A production by CD8⁺ T cells (Figure 4B). CD161, a C-type lectin like receptor, is expressed on CD4⁺ and CD8⁺ T cells, and a subset of NK cells. Expression of CD161 has been associated with T_H17/T_C17 subset as well as mucosal-associated invariant T (MAIT) cells [25, 36]. CD161⁺ T cells have been found in rheumatic joints, psoriatic lesions, and inflammatory bowel disease, indicating an association with pro-inflammatory responses [37]. Due to cell limitations, the expression of CD161 could be determined in only 2 volunteers. We observed that CD161 expression was predominant in quadruple positive CD4⁺ T cells (IL-17A⁺ IL-2⁺ IFN- γ ⁺ TNF- α ⁺) with a similar trend observed in CD8⁺ T cells. Although the interpretation of these results is limited by the number of volunteers tested, it strongly suggests that triple and quadruple cytokine producing populations share some, but not all, the markers typically associated with the T_H17/T_C17 subset.

CCR6 is a molecule associated with homing of lymphocytes and dendritic cells to sites of mucosal inflammation [18]. The ligand for CCR6, CCL-20 (also known as MIP-3 α), is expressed by multiple cell types including pulmonary and intestinal epithelial cells and expression is increased in the presence of pro-inflammatory cytokines [18]. Expression of CCR6 has been associated with T_H17/T_C17 responses and homing of these cells to sites of inflammation [38]. Thus, the expression of CCR6 by some multifunctional IL-17A⁺ T cells described in this manuscript may contribute to the pathogenesis of TSS by directing these cells to mucosal sites, specifically sites of inflammation, contributing further to an ongoing cytokine storm and inflammatory process.

Due to limitations in the number of markers that can be simultaneously detected by flow cytometry, IL-17A is frequently used independently as a marker of T_H17/T_C17 cells. However, taken together, our results indicate that in response to direct TCR stimulation, the majority of IL-17A is produced by multifunctional T cells, some of which do not express all

markers typically associated with the T_H17/T_C17 subset. Therefore, IL-17A is unlikely to serve as a single marker of T_H17/T_C17 subset. Alternatively, the T_H17/T_C17 subsets may not be as well defined as T_H1 and T_H2 subsets, since these cells might functionally downregulate ROR- γ_t expression whilst maintaining IL-17A production in conjunction with other cytokines. It has been proposed that while T_H17 cells are a subset distinct from T_H1 and T_H2 cells, there may be more plasticity than originally thought [39]. In fact, studies have indicated that T_H17 cells can lose expression of ROR- γ_t and express T-bet, a transcription factor typically associated with T_H1 cell subset [39]. Furthermore, during the transition from T_H17 to T_H1 , these cells have been shown to co-produce IL-17A and IFN- γ [31]. We showed that higher percentages of these quadruple positive CD4⁺ T cells (IL-17A⁺ IL-2⁺ IFN- γ ⁺ TNF- α ⁺) co-express the T_H1/T_C1 transcription factor T-bet than the T_H17/T_C17 ROR- γ_t (Figure 6) indicating that these cells are predominantly T_H1/T_C1 -like.

To determine if the multifunctional nature of IL-17A⁺ T cells is unique to direct TCR stimulation by SEB, we compared IL-17A⁺ responses following PHA and PMA/Ionomycin stimulation. PHA is a lectin found in plants that activates T cells by stimulating the phospholipase C (PLC)-inositol-1,4,5-triphosphate system via crosslinking proteins on the lymphocyte surface, including the TCR/CD3 molecule complex [40]. We identified IL-17A production following stimulation with PHA as well as SEB and PMA/Ionomycin (Supplemental Figure 4). Intriguingly, we identified significant differences in the proportion of single positive IL-17A⁺ compared to multifunctional IL-17A⁺ CD4⁺ T cells following stimulation with SEB versus PHA (Supplemental Figure 4). These differences suggest that activation of the TCR directly may trigger a unique multifunctional response, including cells that co-produce IL-17A, IL-2, IFN- γ and/or TNF- α , that is different than that elicited by non-specifically cross-linking proteins on the cell membrane. As discussed previously, multifunctionality has been associated with higher quality T cell responses.

In order to further validate our findings, we used the novel FLOCK analyses. The unsupervised nature of the FLOCK analyses further confirms that multifunctional IL-17A producing populations are present following SEB stimulation. Although the percentages varied between conventional and unsupervised methods, the overall trends were the same. FLOCK uses mathematical algorithms to define populations according to the proximity of a cell/event to a centroid [24]. Populations are differentiated based on the MFI of each marker included in the analysis. It is difficult to determine at present the significance of differences in MFI that may be separating populations since the data are divided into many more populations than would be generated using standard methods of defining markers as positive/negative or even bright/dim/negative. However, given the growing acceptance of the importance that the quantity and combinations of cytokines produced by individual cells appears to have in progression or protection from disease, the absence or presence of defined subsets, particularly those producing high levels of multiple cytokines/chemokines, even if they comprise a small percentage of the total population, might be critically important [32].

5. Conclusions

In sum, through the use of 12 and 13-color multichromatic flow cytometry we have shown that IL-17A is produced by multifunctional cells in response to SEB. These multifunctional cells are triple and quadruple positive and co-produce pro-inflammatory cytokines IL-2, IFN- γ , and/or TNF- α , but do not produce IL-10, MIP-1 β or express CD107a/b. Additionally, a subset of these multifunctional IL-17A producing cells, co-produce IL-22 and express ROR- γ_t , CCR6, and/or CD161. Although some of these multifunctional IL-17A⁺ T cells co-express markers characteristic of the T_H17/T_C17 subset, they also co-express T-bet, a transcription factor associated with the T_H1/T_C1 subset.

SEB is a mediator of non-menstrual TSS and the induction of IL-17A production could play a potential role in immunopathogenesis. In a mouse model of SEB induced TSS, IL-17A has been detected in the serum following SEB challenge [13]. The production of IL-17A in response to SEB, could contribute to the pathophysiology of non-menstrual TSS via the initiation of cytokine cascade. Furthermore, the presence of multifunctional T cells co-producing IL-17A, IL-2, IFN- γ , TNF- α and/or IL-22 may indicate a more robust pro-inflammatory response as multifunctional T cells have been shown to have increased functionality compared to single-cytokine producing cells [32]. Additionally, the combined pro-inflammatory effects of IL-17A and IFN- γ , as well as TNF- α , have been shown to be synergistic [11].

Overall, the results presented in this manuscript clearly emphasize the complexity of the response of human T cell subsets following superantigen (*i.e.*, SEB) activation via the TCR, suggesting that future studies directed to investigate the mechanisms underlying TSS using patient specimens should be focused on defining the role of individual subsets and cytokine-producing cells and correlate these observations with clinical outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. We studied secretion of IL-17A by CD4⁺ and CD8⁺ T cells to direct TCR stimulation
2. IL-17A-secreting T cells are multifunctional, co-expressing IFN- γ , TNF- α and IL-2
3. Multifunctional IL-17A⁺ cells express markers of T_H17/T_C17 and T_H1 subsets
4. Unsupervised FLOCK analysis uncovered additional distinct IL-17A⁺ populations
5. IL-17A multifunctional T cells are likely to play a role in the pathogenesis of TSS

Figure 1. Simultaneous detection of 6 cytokines/chemokines produced by CD4+ and CD8+ T cells in response to SEB stimulation

A & B. Scatter plots showing IL-17A production by CD4+ CD69+ or CD8+ CD69+ memory T cell subsets (T_{CM}, T_N, T_{EM}, T_{EMRA}). Median and interquartile ranges are indicated. N=18. C & D. Scatter plot showing the 10 predominant cytokine production patterns in CD4+ T cells with median and interquartile ranges. Due to space constraints only the top 10 populations are shown. E & F. Scatter plots showing cytokine production patterns for IL-17A+ CD4+ T cells with median and interquartile ranges indicated. In initial studies MIP-1 β antibody was not available. Thus, specimens from 2 volunteers (C & E) were evaluated for the simultaneous production of 5 cytokines (IL-17A, IL-2, IFN- γ , TNF- α and IL-10). Specimens from 5 additional volunteers were evaluated for the above cytokines plus MIP-1 β (D & F). Multifunctionality was analyzed using the FCOM feature of WinList (Verity Software House).

Figure 2. Simultaneous detection of 6 cytokines/chemokines produced by CD8+ T cells in response to SEB stimulation

A & B. Scatter plots showing the 10 predominant CD8+ T cell cytokine production patterns with median and interquartile ranges indicated. Due to space constraints only the top 10 populations are shown. C & D. Scatter plots showing cytokine production patterns for IL-17A+ CD8+ T cells with median and interquartile ranges indicated. As described in the legend to Figure 1, specimens from 2 volunteers (A & C) were not evaluated for MIP-1 β production. Multifunctionality was analyzed using the FCOM feature of WinList (Verity Software House).

Figure 3. Simultaneous measurement of 4 cytokines and CD107a/b in CD4+ and CD8+ T cells in response to SEB stimulation

A & B. Scatter plots showing cytokine profiles and expression of CD107a/b with median and interquartile ranges in CD4+ (A) and CD8+ (B) T cells. Due to space constraints only the top 10 populations are shown. C. Scatter plots of all possible IL-17A+ cytokine production patterns in CD4+ T cells with median and interquartile ranges indicated. N=11.

Figure 4. Co-expression of markers of the “T_H17/T_C17” subset in multifunctional IL-17A+ CD4+ and CD8+ T cells

A & B. Scatter plots showing expression of IL-22 and ROR- γ t by quadruple positive (IL-17A+ IL-2+ IFN- γ + TNF- α +) CD4+ (A) and CD8+ (B) T cells with median and interquartile ranges indicated. N=5.

Figure 5. CD161 expression in IL-17A single positive, triple positive, and quadruple positive CD4+ T cells

Histograms from a representative volunteer showing CD161 expression for A. Total CD4+ CD69+ T cells B. CD4+ CD69+ IL-17A single positive cells (IL-17A+ IL-2- IFN- γ -TNF- α -) C. CD4+ CD69+ IL-17A triple positive cells (IL-17A+ IL-2+ IFN- γ -TNF- α +) and D. CD4+ CD69+ IL-17A quadruple positive cells (IL-17A+ IL-2+ IFN- γ + TNF- α +).

Figure 6. Co-expression of T_H17/T_C17 or T_H1/T_C1 markers by multifunctional IL-17A+ CD4+ T cells

Scatter plot showing expression of CCR6, ROR- γ t, and T-bet by quadruple positive (IL-17A+ IL-2+ IFN- γ + TNF- α +) CD4+ T cells with median values indicated. Values are shown as the percentage of CD4+ CD69+ IL-17A+ IL-2+ IFN- γ + TNF- α + cells co-expressing the CCR6 chemokine receptor or transcription factors ROR- γ t or T-bet. N=3.

Figure 7. Characteristics of 17 distinct CD69+ populations defined by FLOCK
A. Scatter plot depicting the top 10 populations which were positive for the measured cytokines by FLOCK with median and interquartile ranges indicated. B. Positivity based on MFI. – indicates negative, + indicates positive (dim), and ++ indicates positive (bright). IL-17A+ populations are highlighted (population 12 in gold and population 14 in dark red).

Figure 8. IL-17A⁺ multifunctional T cells can be separated into multiple populations with distinct cytokine production patterns by FLOCK analyses

A. Representative volunteer showing 2 populations of IL-17A⁺ multifunctional CD4⁺ T_{EM} cells identified using FLOCK. Population 12 depicted in gold (IL-17A^d IL-2⁺ TNF-α⁺) and population 14 (IL-17A^{br} IL-2⁺ IFN-γ⁺ TNF-α⁺) depicted in dark red.

Table 1
Percentages of CD69+ populations derived by FLOCK in all volunteers

The net percentages (SEB – media control) for each of the 17 CD69+ populations identified by FLOCK (shown in Figure 7) from CD4+ T_{EM} are shown. Volunteers are identified in the left column. N=11. IL-17A+ populations are highlighted (population 12 in gold and population 14 in dark red)

Volunteer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
133	4.92	3.57	0.81	3.99	4.93	3.85	1.78	4.47	8.6	1.12	7.99	0.52	19.7	0.14	1.35	4.69	1.88
134	4.9	2.74	1.46	5.12	3.6	2.55	2.28	5.1	6.35	1.54	6.91	0.94	6.93	0.37	1.33	3.86	3.87
135	5.89	3.25	1.28	5.32	4.85	2.93	2.73	7.2	6.61	1.66	7.24	1.27	9	0.34	1.92	3.86	3.49
202	5.44	2.86	1.69	5.08	4.45	2.92	3.09	5.8	7.23	2.21	7.25	1.52	9.08	0.78	1.74	4.41	3.06
204	3.81	2.24	3.67	3.64	5.04	2.13	5.36	4.93	5.43	4.85	5.36	3.83	6.81	1.5	2.48	3.11	3.18
206	4.63	3.08	1.63	4.15	5.65	2.82	3.55	6.7	6.57	2.58	6.33	1.38	12	0.62	2.17	4.21	2.94
214	3.27	1.22	10.8	4.54	4.57	0.99	7.27	7.95	1.44	2.93	2.42	7.28	3.34	1.05	2.64	2.21	3.79
209	2.67	1.58	5.6	3.12	3.52	0.68	4.46	5.79	2.75	1.23	3.35	3.9	3.04	0.38	2.02	2.62	2.4
215	2.53	0.96	10.5	3.02	4.41	0.95	7.28	5.98	1.28	1.76	2.06	8.06	3.12	1.88	2.9	1.32	2.93
213	2.51	1.13	11.1	2.99	4.29	0.72	5.65	4.51	0.99	4.48	1.88	4.84	3.31	1.1	2.55	1.29	4
217	3.44	1.96	6.96	4.21	6.12	1.52	7.63	10.3	2.3	2.21	3.51	6.18	4.64	1.12	3.12	2.86	2.52