Temporal Sequence and Kinetics of Proinflammatory and Anti-Inflammatory Cytokine Secretion Induced by Toxic Shock Syndrome Toxin 1 in Human Peripheral Blood Mononuclear Cells

WINNIE W. S. KUM, SCOTT B. CAMERON, RYAN W. Y. HUNG, SHIRIN KALYAN, AND ANTHONY W. CHOW*

Division of Infectious Diseases, Departments of Medicine, Microbiology, and Immunology, University of British Columbia and Vancouver Hospital Health Sciences Center, Vancouver, British Columbia, Canada

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The staphylococcal superantigen toxic shock syndrome toxin 1 (TSST-1) induces massive cytokine production, which is believed to be the key factor in the pathogenesis of TSS. The temporal sequence and kinetics of both proinflammatory and anti-inflammatory cytokines induced by TSST-1 in human peripheral blood mononuclear cells were investigated. A panel of loss-of-function single-amino-acid-substitution mutants of TSST-1, previously demonstrated to be defective in either major histocompatibility complex (MHC) class II binding (G31R) or T-cell receptor (TCR) interaction (H135A, S14N), was studied in parallel to further elucidate the mechanisms of cytokine secretion. Wild-type recombinant (WT r) TSST-1 induced a biphasic pattern of cytokine secretion: an early phase with rapid release of proinflammatory cytokines (especially gamma interferon, interleukin-2 [IL-2], and tumor necrosis factor alpha [TNF-α]) within 3 to 4 h poststimulation, and a later phase with more gradual production of both proinflammatory (IL-1β, IL-12, and TNF-β) and anti-inflammatory (IL-6, IL-10) cytokines within 16 to 72 h poststimulation. G31R, which is defective in MHC class II binding, induced a cytokine profile similar to that of WT rTSST-1, except that secretion of the early-phase proinflammatory cytokines was delayed and production of IL-1β and IL-12 was markedly reduced. In contrast, mutant toxins defective in TCR interaction either demonstrated complete absence of any cytokine secretion during the entire observation period (H135A) or resulted in complete abolishment of IL-2 and other early-phase proinflammatory cytokines, while secretion of IL-10 appeared unaffected (S14N). Neither WT rTSST-1 nor the mutant toxins induced IL-4 or transforming growth factor β. Our data indicate that effective TCR interaction is critical for the induction of the early-phase proinflammatory cytokine response, thus underscoring the importance of T-cell signaling in TSS.

Cytokines are the primary modulators of the immune response, and have either proinflammatory or anti-inflammatory functions (43). They are derived either from APCs or from T-helper cells, which can be categorized into two major subsets, Th1 and Th2, based on their cytokine production profiles (34). Th1 effector cells produce predominantly proinflammatory cytokines such as IFN-γ, IL-2, TNF-α, and TNF-β, which are associated with cell-mediated immunity. Th2 effector cells produce largely anti-inflammatory cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13, which are associated with humoral immunity (1, 33). Both T-cell subsets are capable of cross-regulating and suppressing each other through a complicated network of cytokine-mediated signaling (1, 9, 37). For example, IFN-γ produced by Th1 cells inhibits the development of Th2 cells (11), whereas IL-4 and IL-10 produced by Th2 cells inhibit Th1 development (33, 47). IL-6 may possess both proinflammatory and anti-inflammatory effects depending on the particular model system being studied (14). There is evidence for the presence and activity of each of these Th subsets during the immune response to bacterial superantigens (9, 12, 30, 31, 50). In the present study, we sequentially monitored the Th1 and Th2 cytokine profiles following TSST-1 stimulation of human PBMC in vitro. To further clarify the possible mechanism of TSST-1-induced cytokine production, a panel of loss-of-function, single-amino-acid-substitution TSST-1 mutant toxins...
were studied in parallel. These mutant toxins include G31R, which was previously found to be defective in MHC-II binding (24), as well as S14N (W. W. S. Kum, R. W. Y. Hung, S. B. Cameron, and A. W. Chow, submitted for publication) and H135A (6, 7), which were previously found to be defective in TCR interaction.

**MATERIALS AND METHODS**

**Purification of WT rTSST-1 and mutant toxins.** Wild-type recombinant TSST-1 (WT rTSST-1) and mutant toxins S14N, G31R, and H135A were obtained by random and site-directed mutagenesis as described previously (24). WT and mutant toxin genes were transformed into Staphylococcus aureus RN4220, and expressed toxins were purified from lipopolysaccharide (LPS)-free culture supernatants using a combination of preparative isoelectric focusing and chromatofocusing as reported previously (23). Toxin purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 14% acrylamide gel and silver staining, and LPS activity was monitored by the *Laminus amoebocyte lysate* test (sensitivity limit, 10 pg per ml) (23).

**Preparation of human PBMC and culture conditions.** Fresh human PBMC from random healthy adult donors were obtained by centrifugation of leukopheresis packs over Histopaque 1.077 (Pharmacia Fine Chemicals, Dorval, Quebec, Canada). Approximately 2 × 10^6 human PBMC were cultured in growth medium containing RPMI 1640 medium (Stem Cell Vancouver, British Columbia, Canada) supplemented with 10% fetal bovine serum (heat inactivated at 56°C for 30 min; HyClone Laboratories, Inc., Logan, Utah), 2 mM L-glutamine (GIBCO BRL, Burlington, Ontario, Canada) and 20 μg of polymyxin B sulfate (to neutralize any possible LPS contamination) (Sigma Chemical Co., St. Louis, Mo.) per ml in 24-well tissue culture plates (Falcon Labware; Becton Dickinson Canada Inc., Mississauga, Ontario, Canada) and incubated at 37°C in 5% CO2 with WT rTSST-1 or mutant toxins S14N, G31R, and H135A at a concentration of 1 nM. WT rTSST-1 and the mutant toxins S14N and G31R stimulated equivalent maximal levels of T-cell proliferation at this concentration as determined by [3H]thymidine incorporation (24). In order to study the kinetic profiles of cytokine secretion, cells were exposed to the toxins over a period of 3 days. Culture supernatants were collected at 0.5 h, at 1-h intervals from 1 to 6 h, at 2-h intervals from 8 to 20 h, at 48 h, and at 72 h. Stimulated supernatants were microcentrifuged at 800 × g for 5 min, transferred to fresh vials, and frozen at −70°C until analysis.

**Cytokine assays.** Culture supernatants from cells stimulated with WT rTSST-1, S14N, G31R, or H135A at various time intervals were assayed for different cytokines by enzyme-linked immunosorbent assay (ELISA), using commercial sandwich ELISA kits containing recombinant human cytokine standards, murine monoclonal capture antibodies, and biotinylated goat anti-human cytokine detecting antibodies as described previously (22, 46). The sources of various ELISA kits for the different cytokine assays (and their sensitivity limits) are as follows: (i) TNF-α (62 pg/ml), TNF-β (16 pg/ml), IFN-γ (62 pg/ml), IL-1β (125 pg/ml), IL-2 (125 pg/ml), IL-6 (20 pg/ml), and IL-12 (31 pg/ml) from R&D Systems, Minneapolis, Minn.; (ii) IL-4 (62 pg/ml), IL-10 (62 pg/ml), and transforming growth factor β (TGF-β) (62 pg/ml) from Pharmingen, San Diego, Calif. Data analysis. The GraphPad PRISM version 3.0 software (GraphPad Software, Inc., San Diego, Calif.) was used for data analysis. Cytokine assays by ELISA were determined in duplicate, and data were obtained from three different donors. Time-dependent changes in cytokine levels between each toxin treatment group were assessed by one-way analysis of variance with repeated measures. Bonferroni’s test was used for multiple comparisons between the different toxin treatment groups. Differences were considered significant if the 2-tailed probability of the null hypothesis was less than five percent (P < 0.05).

### RESULTS AND DISCUSSION

**Toxin purity and characterization.** All toxins were LPS-free and migrated as single protein bands on SDS-PAGE with approximate molecular masses of 22 kDa, and each reacted with rabbit polyclonal anti-TSST-1 antibody by Western immunoblotting (data not shown). Previously reported biologic properties of these toxins including MHC-class II binding constants, maximal T-cell proliferation (22, 24), Vβ2-specific T-cell proliferation, and TCR down-regulation in human PBMC (Kum et al., submitted) are summarized in Table 1.

**Secretion profiles of the early-phase (within 3 to 4 h) cytokines.** Three proinflammatory cytokines, IFN-γ, IL-2, and TNF-α, were detected in culture supernatants within 3 to 4 h following stimulation with WT rTSST-1 (Fig. 1A to C).

(i) **IFN-γ.** IFN-γ was induced by WT rTSST-1 very early in human PBMC, being first detectable in culture supernatants within 3 h, increasing rapidly until 8 h, and more gradually through 48 h (Fig. 1A). The MHC-II-binding-defective mutant toxin G31R demonstrated a kinetic pattern similar to that of WT rTSST-1 but with a slightly delayed onset (~5 h) and blunted response throughout the observation period. In contrast, the mutant toxins with defective TCR interaction either did not induce any IFN-γ secretion throughout the entire 72-h period (H135A) or demonstrated a markedly delayed response at 48 h with no detectable IFN-γ secretion in the early phase (S14N) (P < 0.001 compared to WT rTSST-1) (Fig. 1A).

(ii) **IL-2.** Human PBMC stimulated with WT rTSST-1 also exhibited a biphasic IL-2 response, which was first detectable at 3 h, with rapid increase in the first 8 h, and a more gradual increase through 48 h (Fig. 1B). G31R induced a similar secretion profile but with a delayed onset at ~6 h. Surprisingly, even though mutant toxin S14N had retained the ability to induce Vβ2-specific T-cell proliferation in a previous study (Kum et al., submitted), it did not induce any detectable IL-2 production throughout the 72-h observation period. H135A again did not induce any cytokines, as shown by a comparison with RPMI controls.

(iii) **TNF-α.** TNF-α was first detectable at ~3 to 4 h following stimulation with WT rTSST-1, and its secretion profile also appeared to follow a biphasic course, with a rapid rate of production during the early phase until 8 h and a more gradual but sustained rate of production thereafter through 48 h (Fig. 1C). G31R induced similar levels of TNF-α but with a slightly
FIG. 1. Detection of proinflammatory and anti-inflammatory cytokines by ELISA in human PBMC stimulated by WT rTSST-1, and loss-of-function single-amino-acid-substitution mutant toxins G31R (MHC-II binding defective) and S14N and H135A (both TCR interaction defective). Results (means ± standard errors of the means) were from three different donors. Panels: A, IFN-γ; B, IL-2; C, TNF-α; D, IL-1β; E, IL-6; F, TNF-β; G, IL-10; H, IL-12.
delayed onset (~8 h). The production of TNF-α induced by S14N was markedly delayed (detectable only at 48 h), and quantifiable levels were markedly diminished compared to those observed with WT rTSST-1 (P < 0.001). H135A again did not induce any cytokines at any time.

**Secretion profiles of the late-phase (16 to 48 h) cytokines.** Most of the other cytokines induced by WT rTSST-1 in human PBMC were detected only at ~16 h or later (Fig. 1D to H). These included both proinflammatory (IL-1β, TNF-β, IL-12) as well as anti-inflammatory (IL-6, IL-10) cytokines. G31R induced a similar cytokine profile, except that production of IL-1β and IL-12 was markedly reduced. H135A again did not induce any cytokines at any time. With the exception of IL-10, S14N induced lower levels of all cytokines than did G31R and WT rTSST-1 during the 72-h observation period.

(i) **IL-1β.** Secretion of the Th1 cytokine IL-1β by WT rTSST-1 in human PBMC was not observed until ~16 h, attaining peak levels at 48 h (Fig. 1D). IL-1β secretion was induced by both mutant toxins S14N and G31R, with similar kinetic profiles, although the concentrations induced by each of these were markedly reduced compared to that induced by WT rTSST-1 (P < 0.001).

(ii) **IL-6.** WT rTSST-1 and G31R induced similar secretion profiles of IL-6, which was first detected at ~16 h, reaching peak levels at 48 h (Fig. 1E). Significantly reduced production of IL-6 was induced by S14N compared to that induced by WT rTSST-1, being detectable only at ~20 h and with markedly diminished levels at 48 h (P < 0.001) (Fig. 1E).

(iii) **TNF-β.** Both WT rTSST-1 and mutant toxin G31R induced the secretion of TNF-β, with similar kinetic profiles, being detectable only after 24 h followed by a gradual increase in concentration at 48 to 72 h (Fig. 1F). The mutant toxin S14N induced TNF-β with a kinetic profile similar to those of WT rTSST-1 and G31R, although slightly reduced in comparison.

(iv) **IL-10.** Secretion levels of the Th2 anti-inflammatory cytokine IL-10 in human PBMC were similar for WT rTSST-1 and G31R, being detectable only at ~48 h, followed by a gradual decline in concentration at 72 h (Fig. 1G). In contrast, the induction of IL-10 by S14N continued to increase at 72 h.

(v) **IL-12.** Like those of TNF-β and IL-10, secretions of the proinflammatory cytokine IL-12 in human PBMC stimulated with WT rTSST-1, G31R, and S14N were not observed until ~48 h (Fig. 1H). The IL-12 responses to both G31R and S14N were markedly blunted compared to what was observed with WT rTSST-1 (P < 0.01), with G31R producing a higher level than S14N.

**Absence of detectable anti-inflammatory cytokines IL-4 and TGF-β.** Th2 anti-inflammatory cytokines IL-4 and TGF-β were not detected at any time in any of the culture supernatants stimulated with either WT rTSST-1, G31R, S14N, or H135A, even though the assays had acceptable sensitivity limits for detection (62 pg/ml for each cytokine).

In the present study, the temporal sequence and kinetic profiles of the secretion of several proinflammatory and anti-inflammatory cytokines induced by WT rTSST-1 and three well-characterized loss-of-function, single-amino-acid-substitution mutant toxins in human PBMC were examined in detail. Collectively, the cytokine induction profiles of WT rTSST-1 appeared to follow a biphasic pattern, with an early phase of rapidly released Th1 proinflammatory cytokines including IFN-γ, IL-2, and TNF-α, which were detectable within 3 to 4 h after stimulation, and a later-phase secretion of both Th1 proinflammatory (IL-1β, IL-12, and TNF-β) as well as Th2 anti-inflammatory (IL-6 and IL-10) cytokines, which were detectable only after 16 to 24 h, with peak levels following 48 to 72 h poststimulation (Fig. 1A to H). Unfortunately, we were unable to extend our observations beyond 72 h due to loss of viability of human PBMC without replenishment of fresh culture medium, which would have confounded the interpretation of cytokine determinations. Nevertheless, our finding that WT rTSST-1 induced an early and vigorous Th1 cytokine response characterized by the rapid secretion of IFN-γ, IL-2, and TNF-α within 3 to 4 h poststimulation agrees with prior observations reported by Miethke et al. (29, 30) and others (26, 35, 48). This early-phase proinflammatory cytokine release is considered a key determinant leading to TSST-1-induced lethality in the murine model of TSS (30). Since they were detected in culture supernatants within 3 to 4 h after TSST-1 stimulation, too soon for de novo gene transcription-translation and protein synthesis to complete, we surmised that the detection of these early-phase proinflammatory cytokines was due to the rapid release of preformed rather than newly synthesized cytokines from human PBMC following TSST-1 stimulation. To verify that this was indeed the case would have required further studies by intracellular cytokine staining before and immediately following TSST-1 stimulation, which unfortunately were not performed. However, that this possibility exists is suggested by earlier studies which demonstrated the presence of presynthesized TNF (5, 15) and IL-2 (44) in human PBMC. The release of these early-phase proinflammatory cytokines was likely followed by the sequential synthesis and sustained secretion of a variety of other cytokines, both proinflammatory (IFN-γ, IL-2, TNF-α, IL-1β, and IL-12) and anti-inflammatory (IL-6 and IL-10), that were detected only after 16 to 24 h poststimulation. The late-phase secretion of both IFN-γ and TNF-α by human PBMC following TSST-1 stimulation has also been observed by others (44).

Both proinflammatory cytokines TNF-β and IL-12 were detected only during the later phase (~48 h) after TSST-1 stimulation. IL-12 is induced in activated APCs in response to the Th1 cytokine IFN-γ (4), which is reciprocally induced in T cells by IL-12 (27, 48). Furthermore, exogenous IL-12 also appears to up-regulate the anti-inflammatory cytokine IL-10 (28, 32). Thus, the production of IL-12 in human PBMC following TSST-1 stimulation may have been responsible for the late and sustained release of both IFN-γ and IL-10 observed in the present study.

Of interest, among the Th2 anti-inflammatory cytokines examined in the present study, only IL-6 and IL-10 but not IL-4 or TGF-β were detected in human PBMC following TSST-1 stimulation. These Th2 anti-inflammatory cytokines are believed to down-regulate the cellular Th1 effector functions following chronic superantigen stimulation (9, 13, 36). For example, exogenous IL-10 and IL-4 are both capable of inhibiting IFN-γ and other Th1 cytokines induced by TSST-1 in human PBMC, although IL-4 is much less effective than IL-10 in this activity (10, 21). IL-10 also inhibits the induction of IL-12 (2). Thus, IL-10 appears to provoke negative-feedback inhibition of an overwhelming Th1 proinflammatory response to TSST-1. In contrast to the report by Krakauer (21), who
noted only low levels of IL-10 in human PBMC following stimulation with TSST-1, we detected high levels of IL-10 in the present study, but only after prolonged stimulation (≈48 h) with TSST-1. In this regard, repeated stimulation with the staphylococcal enterotoxins A and B, SEA and SEB, respectively, has been associated with the generation of a newly characterized T-cell subset known as regulatory T cells (Tr-1), which produce little or no IL-4 but predominantly IL-10 with or without TGF-β (9, 31, 36, 50). Whether the high-level production of IL-10 following prolonged TSST-1 stimulation observed in the present study was also induced by the activation of immunoregulatory Tr-1 cells remains to be determined. In agreement with what was observed by Krakauer (21), we were unable to detect IL-4 in human PBMC following TSST-1 stimulation. Interestingly, although the induction of IL-4 mRNA in human PBMC can be readily detected following stimulation with all staphylococcal superantigens including TSST-1 (25, 42), the detection of IL-4 in stimulated human PBMC has variable for different superantigens and in different studies for the same superantigens. Thus, whereas both Krakauer (20, 21) and Rink et al. (41) were unable to detect IL-4 in culture supernatants of human PBMC after stimulation with either TSST-1, SEA, or SEB, Lagoo et al. (25) reported “significant levels” of IL-4 following primary stimulation of human T cells and monocytes with SEA and SEB. The reason for this discrepancy is unclear. One possible variable which could account for these disparate results from different laboratories could be the purity of the superantigens used in the different studies (23). In the present study, recombinant TSST-1 devoid of LPS or other potential contaminants was utilized, but neither IL-4 nor TGF-β production could be detected by ELISA (sensitivity limit, 62 pg/ml for each assay) despite prolonged stimulation for 72 h in human PBMC.

The availability of a panel of well-defined, loss-of-function, single-amino-acid-substitution TSST-1 mutant toxins in the present study provided a unique opportunity to further elucidate the possible mechanisms of TSST-1-induced proinflammatory as well as anti-inflammatory cytokine production in human PBMC. Since secretion of the early-phase Th1 proinflammatory cytokines in human PBMC was completely absent when stimulated by TSST-1 mutant toxins defective in TCR interaction (either H135A or S14N) but less affected by the TSST-1 mutant toxin defective in MHC-II binding (G31R), we conclude that effective TCR interaction is critical for the induction of the early Th1 proinflammatory cytokine response, thus underscoring the importance of T-cell signaling in TSS. The complete absence of detectable IL-2 production by the TCR-defective TSST-1 mutant S14N was somewhat surprising, since S14N was previously found to retain the ability to induce Vβ2-specific T-cell proliferation (Kum et al., submitted). This suggests that the N-terminal serine residue (S14) of TSST-1 may be essential for TSST-1-induced IL-2 production and that both IL-2-dependent and -independent mechanisms may be present for TSST-1-induced Vβ2-specific T-cell proliferation in human PBMC. IL-2-independent activation and proliferation of human T cells has been previously described following CD28 and CD154 costimulation of T cells and CD80/CD86 (B7.1-B7.2) costimulation of the APC (8). In this regard, previous studies of costimulatory molecule expression in human PBMC following S14N stimulation revealed that the surface expression of CD28 on T cells was unaffected, while that of CD154 was markedly reduced (14% compared to WT rTSST-1) and that of CD80/CD86 on human APCs was only minimally decreased (Kum et al., submitted).

Secretion levels of the proinflammatory monokine IL-1β by human PBMC stimulated by mutant toxins G31R and S14N were similar to those seen with WT rTSST-1, although their levels were significantly reduced (P < 0.05) (Fig. 1D). In contrast to those of IL-1β, the kinetic profiles of IL-6 production induced by G31R were almost identical to that induced by WT rTSST-1, while its secretion following S14N stimulation was significantly reduced (P < 0.05) (Fig. 1E). IL-6 is produced by both activated T cells and monocytes following superantigen stimulation and may possess both proinflammatory and anti-inflammatory effector functions depending on the particular model system being studied (14). Similarly, the production levels of IL-12 induced by mutant toxins G31R and S14N were markedly reduced in comparison to that induced by WT rTSST-1 (P < 0.05) (Fig. 1H). The markedly decreased production of IL-12 following S14N stimulation may be attributed to the complete lack of the early-phase Th1 cytokine IFN-γ, which is known to secondarily induce the expression of IL-12 in activated APCs (4, 48).

In contrast to the proinflammatory cytokines which were markedly reduced in culture supernatants stimulated by both mutant toxins G31R and S14N, production of the anti-inflammatory cytokine IL-10 was equivalent to that observed with WT rTSST-1 at 48 h poststimulation. Curiously, IL-10 levels in the supernatants stimulated by S14N appeared to continue to increase by 72 h in contrast to those stimulated by both WT rTSST-1 and G31R (P < 0.05) (Fig. 1G). Possible mechanisms for the sustained release of IL-10 by S14N remain unclear and are currently under investigation.

In conclusion, the temporal sequence and kinetic profiles of both Th1 and Th2 cytokines induced by WT rTSST-1 in human PBMC reported here parallel the in vivo observations both in mice (29, 30) and in rabbits (16, 39) and thus may be a key determinant of the ultimate lethal response induced by TSST-1. The use of a panel of loss-of-function single-amino-acid-substitution TSST-1 mutant toxins further supports this notion. The observation that a TCR-defective TSST-1 mutant, S14N, failed to induce any IL-2 production but still retained the capacity for sustained and heightened production of the anti-inflammatory cytokine IL-10 is particularly noteworthy. These mutant toxins may prove invaluable for future studies aimed at uncovering the critical signaling pathways induced by TSST-1 and the pathogenesis of TSS.

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