

Staphylococcal toxic shock syndrome toxin-1 inhibits monocyte apoptosis

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Background: Chronic atopic dermatitis (AD) lesions are associated with colonization by exotoxin-producing *Staphylococcus aureus*. Evidence suggests that cytokine production in AD, particularly of GM-CSF, prolongs survival of both peripheral blood monocytes and dermal monocyte-macrophages, the predominate inflammatory cell in lesions caused by chronic AD. **Objective:** We sought to determine whether the staphylococcal exotoxin, toxic shock syndrome toxin-1 (TSST-1), could stimulate prosurvival cytokine production in monocytes and thereby inhibit apoptosis.

Methods: Cultures of peripheral blood monocytes from normal donors and subjects with AD were incubated with various concentrations of TSST-1, and the incidence of apoptosis was assessed by examining cytospin preparations and the appearance of hypodiploid DNA in the flow cytometer. Culture supernatants were analyzed for GM-CSF, IL-1 β , and TNF- α by ELISA.

Results: TSST-1, in a concentration-dependent manner starting at 0.1 pg/mL, significantly inhibited monocyte apoptosis and resulted in the production of the prosurvival cytokines GM-CSF, IL-1 β , and TNF- α . In coculture conditions with conditioned media from TSST-1-stimulated monocytes, with or without neutralizing antibody to the various cytokines, the data show GM-CSF production was responsible for the inhibition of apoptosis.

Conclusions: The data strongly suggest that staphylococcal exotoxins known to colonize skin lesions on patients with chronic AD may induce the production of GM-CSF, resulting in inhibition of monocyte-macrophage apoptosis, and thereby contribute to the chronicity of this inflammatory disease. (J Allergy Clin Immunol 1999;103:895-900.)

Key words: Monocyte, apoptosis, staphylococcal exotoxins, atopic dermatitis, granulocyte-macrophage colony-stimulating factor

Atopic dermatitis (AD) is a common chronic inflammatory skin disease, affecting nearly 15% of children. Though a family or personal history of atopy is strongly

Abbreviations used

AD: Atopic dermatitis
TSST-1: Toxic shock syndrome toxin-1

associated with AD, the underlying mechanism or mechanisms for chronic AD remain elusive despite considerable investigation.¹ Whereas early lesions are characterized by dermal infiltration of activated T lymphocytes, chronic lesions are associated with dermal infiltration of activated monocyte-macrophages.²⁻⁴ In situ cytokine patterns are likewise known to vary depending on the acuity of the AD skin lesions. Acute AD lesions are associated with a predominance of IL-4, whereas chronic AD lesions express high levels of IL-5 and GM-CSF,^{4,5} cytokines that stimulate and enhance survival of eosinophils and monocytes, respectively. Recently, cultures of circulating monocytes from patients with chronic AD have been shown to have a reduced incidence of apoptosis (programmed cell death) when compared with monocyte cultures from normal individuals.⁴ Furthermore, experiments demonstrated that normal monocytes incubated with supernatants obtained from AD monocytes exhibited significant inhibition of apoptosis, an effect that could be ablated by a neutralizing antibody to GM-CSF. Thus we hypothesize that enhanced survival of both circulating and lesional monocyte-macrophages may contribute to the establishment and chronicity of inflammation. Conversely, apoptosis of effector cells may contribute to the control or resolution of inflammation,^{4,6-8} and understanding mechanisms governing apoptosis may offer new therapies for inflammatory disorders such as AD.

The underlying stimulus for GM-CSF mRNA expression in chronic AD lesions and increased GM-CSF production of AD monocytes is unknown. Colonization with *Staphylococcus aureus* is found in over 90% of AD skin lesions,⁹ and production of exotoxins (eg, toxic shock syndrome toxin-1 [TSST-1]) has been reported in the majority of isolates from patients with AD.¹⁰ Staphylococcal exotoxins are known to act as superantigens by causing marked widespread immune activation, including stimulation of monocyte-macrophages.¹¹ As such, staphylococcal exotoxins are thought to contribute to skin inflammation in AD.^{9,10} Therefore we hypothesized that TSST-1 could stimulate prosurvival cytokine production, particularly GM-CSF production, in monocytes thereby inhibiting apoptosis.

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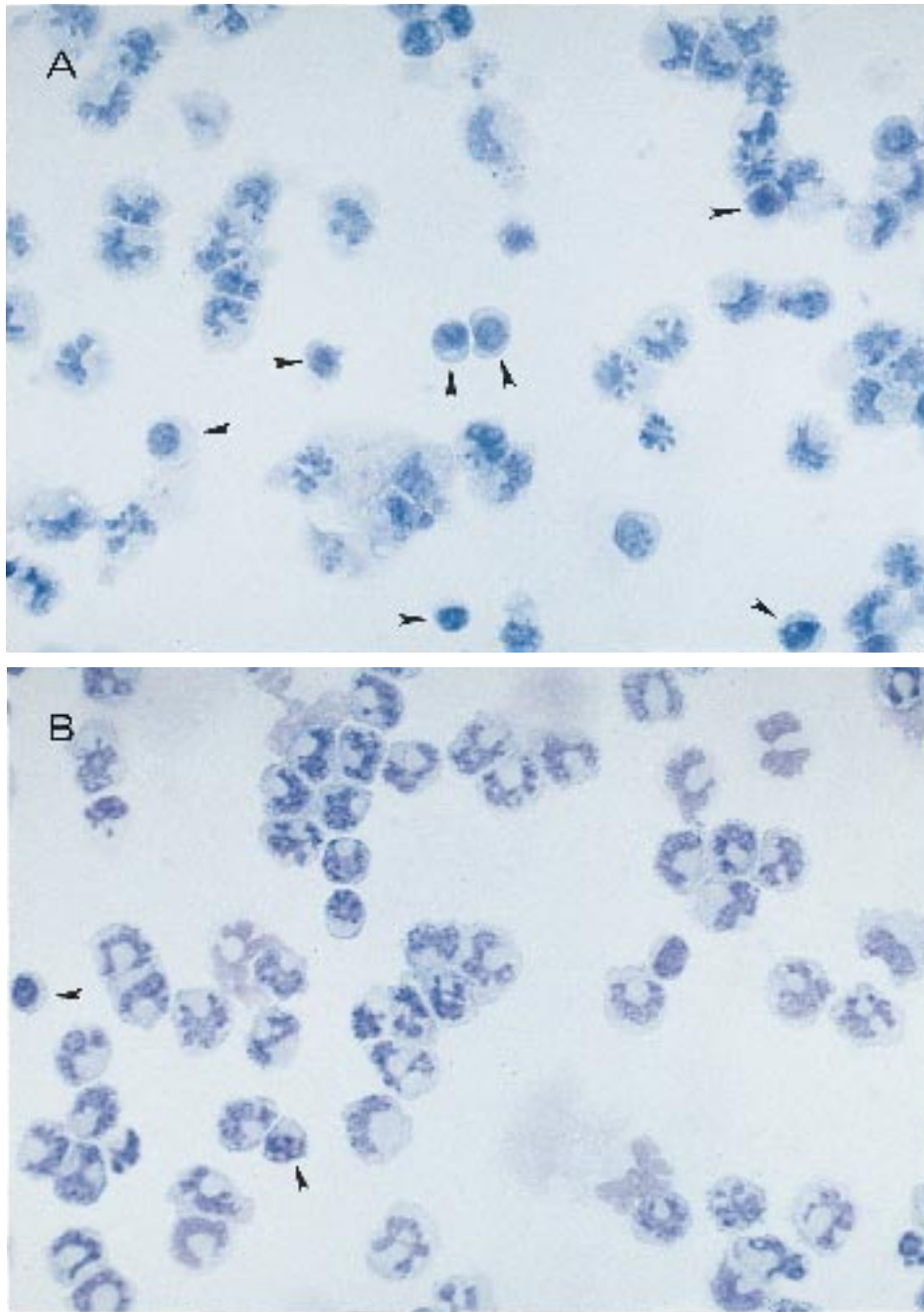


FIG 1. Photomicrograph of apoptotic and nonapoptotic normal monocytes after 24 hours of culture in the absence (**A**) and presence (**B**) of TSST-1 (1 ng/mL). Note that apoptotic monocytes (*arrowheads*) demonstrate loss of cytoplasm and nuclear condensation (magnification, $\times 100$).

METHODS

Monocyte culture and coculture conditions

All materials, procedures, and study populations have been described in detail previously unless otherwise noted below and have the approval of the institutional review board.⁴ Monocytes were isolated from normal and chronic subjects with AD and were cultured in supplemented Dulbecco's modified Eagle's medium with human serum albumin (0.1%) in the presence and absence of TSST-1 (Toxin

Technology, Sarasota, Fla). Cells were harvested at 24 and 48 hours to determine the incidence of apoptosis (see below). Culture supernatants were collected at 24 hours for quantification by ELISA for human (h)GM-CSF (Endogen, Woburn, Mass), hIL-1 β , and hTNF- α (R & D Systems, Minneapolis, Minn). "LPS-free" human serum albumin was obtained from Intergen (Purchase, NY). Because LPS can inhibit monocyte apoptosis and result in activation, every effort was made to minimize exposure to LPS during monocyte isolation, and assay procedures. Plasticware, and reagents were tested for the

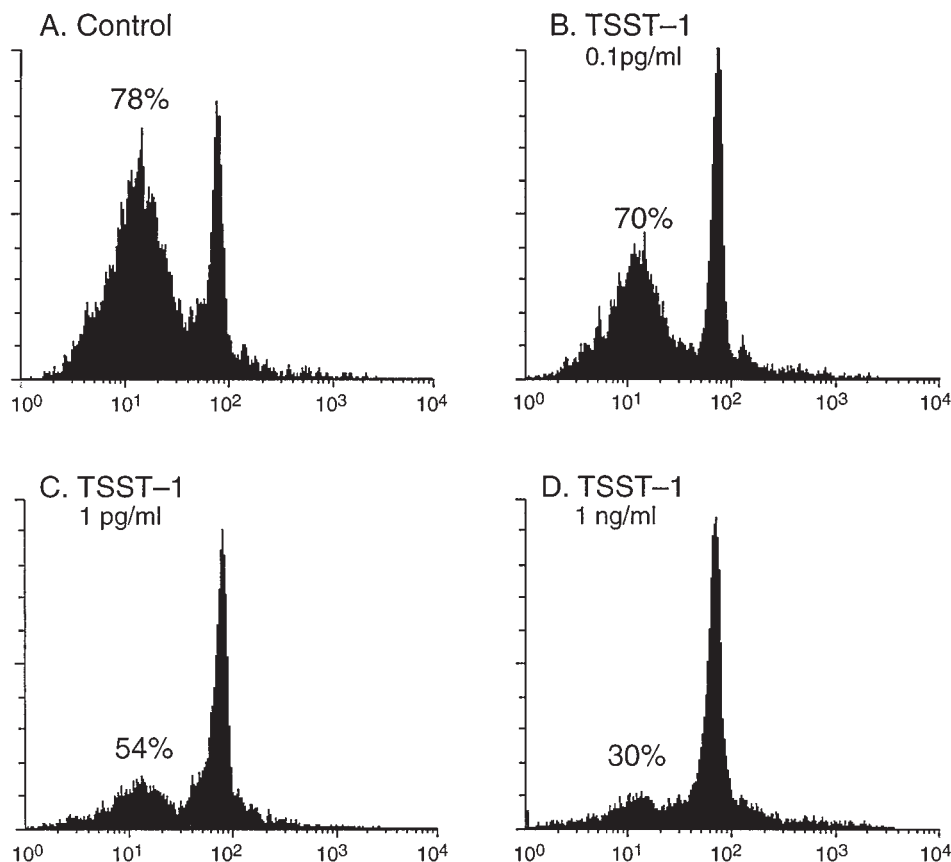


FIG 2. Histograms demonstrating apoptotic normal monocytes identified by the appearance of hypodiploid DNA after 48 hours of culture in the absence (**A**) and presence of TSST-1 0.1 pg/mL (**B**), 1.0 pg/mL (**C**), and 1 ng/mL (**D**). Percent of cells having undergone characteristic DNA fragmentation is shown for each condition. Experiment is representative of 6 experiments.

presence of LPS with the Limulus amoebocyte lysate kit (Associates of Cape Cod, Woods Hole, Mass), which in all cases was less than 0.1 ng/mL LPS, a level at which inhibition of monocyte apoptosis¹² or activation¹³ is not readily demonstrated.

For coculture experiments, conditioned media supernatants were harvested from normal monocytes cultured with or without TSST-1 (1 ng/mL) for 24 hours. The harvested media were treated for 60 minutes with neutralizing antibodies to TSST-1, with or without neutralizing antibody to hGM-CSF, hIL-1 β , and hTNF- α (Genzyme, Cambridge, Mass) singly or in combination used at approximately 2- to 5-fold excess. Conditioned media were then treated with 50 μ L of protein G agarose beads (Pharmacia Biotech, Piscataway, NJ) for 30 minutes at 4°C for the removal of antibodies by centrifugation. Conditioned media was then diluted 1:1 with fresh media for culturing with freshly isolated normal monocytes for 24 hours.

Determining the incidence of apoptosis

The incidence of apoptosis at 24 and 48 hours was determined by 2 methods: analysis of morphology (forward vs side scatter)⁴ and examination of the appearance of degraded hypodiploid DNA by flow cytometry. For the determination of hypodiploid DNA, monocytes were pelleted, washed, and resuspended in PBS (pH 7.4), which was then brought to 80% ethanol. The cells were then held at -20°C overnight and then were pelleted, treated with 200 ng/mL RNase A for 30 minutes at 37°C, and stained with propidium iodide (500

ng/mL). The monocytes were then incubated at 4°C for at least 2 hours before analysis in the flow cytometer. These measures of apoptosis are highly correlated and have been previously verified by light microscopy of sorted cell populations and DNA agarose gel electrophoresis demonstrating 200-bp fragmentation.⁴ For statistical comparisons of normal and AD monocyte apoptosis, the natural log transformation of the percentage of apoptotic cells was used to produce a more normal data distribution. A repeated-measures ANOVA and Fisher's protected multiple comparisons procedure were used at *P* values of less than .05 to determine significant differences.

RESULTS

As has been previously shown, peripheral blood monocytes from normal donors undergo apoptosis over 48 hours when placed in cultures without stimulation.⁴ Apoptotic cells can be distinguished from nonapoptotic cells by their shrunken appearance, with marked loss of cytoplasm and nuclear condensation (Fig 1, A), and by their diminished forward and side scatter in the flow cytometer. These morphologic changes are accompanied by the appearance of fragmented, hypodiploid DNA detected by permeabilization and staining of the cells with propidium iodide.⁴ Importantly, the addition of TSST-1 to cultures significantly inhibited the percentage

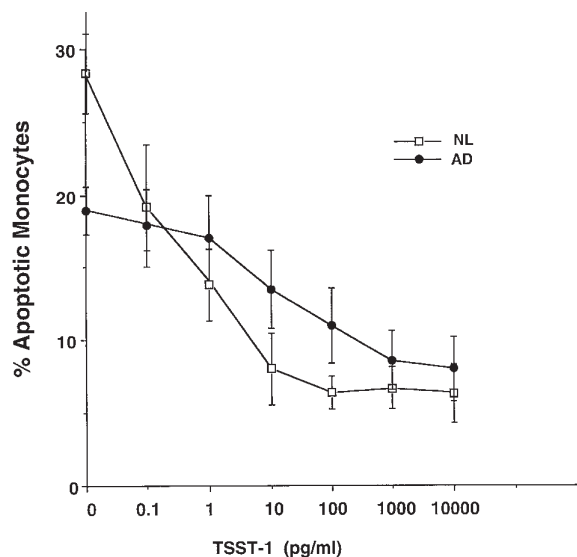


FIG 3. TSST-1 inhibits apoptosis of cultured monocytes. Monocytes from normal subjects (NL, $n = 7$) and subjects with AD ($n = 7$) were cultured for 24 hours in the presence and absence of various concentrations of TSST-1. The incidence of baseline apoptosis was significantly less in chronic AD monocyte cultures than normal monocyte cultures (means of 18.9% and 28.3%, respectively, $P < .01$). A significant concentration-dependent inhibition of apoptosis was seen in both groups, and the response of normal cultures was of greater magnitude and slope than the response of AD cultures (interactive effect), as determined by repeated-measures ANOVA ($P < .02$). Data are represented as means $\pm$ SEM.

of cells undergoing apoptosis in a concentration-dependent manner (Figs 1, B, and 2). This effect on normal monocytes was evident with the addition of TSST-1 at concentrations as low as 0.1 pg/mL, at which the degree of inhibition of apoptosis was similar to that seen in baseline cultures of monocytes from patients with chronic AD after 24 hours of incubation (Fig 3). Similarly, addition of TSST-1 to monocytes from patients with chronic AD significantly decreased the incidence of apoptosis in a concentration-dependent manner starting at 100 pg/mL (Fig 3). The response to TSST-1 was significantly less (rate and magnitude) in AD monocytes compared with normal monocytes at 24 hours ($P < .02$), suggesting possible prior exposure of AD monocytes to TSST-1.

Because GM-CSF is known to reduce monocyte apoptosis and is elevated in AD,⁴ its production was measured after 24 hours' incubation of normal monocytes with TSST-1. As predicted, TSST-1 induced GM-CSF production in a concentration-dependent manner ($P < .001$), with detection starting at 0.1 pg/mL TSST-1 (Fig 4). Because delayed apoptosis of peripheral blood monocytes can also be shown in response to stimulation with TNF- α and IL-1 β ,¹² both were measured after treatment of cultures with TSST-1. As with GM-CSF, both TNF- α and IL-1 β were produced in a concentration-dependent manner (Fig 4).

In an attempt to determine which of the cytokines was mediating the prosurvival effect of TSST-1, neutralizing antibodies to the various cytokines (at 2- to 5-fold

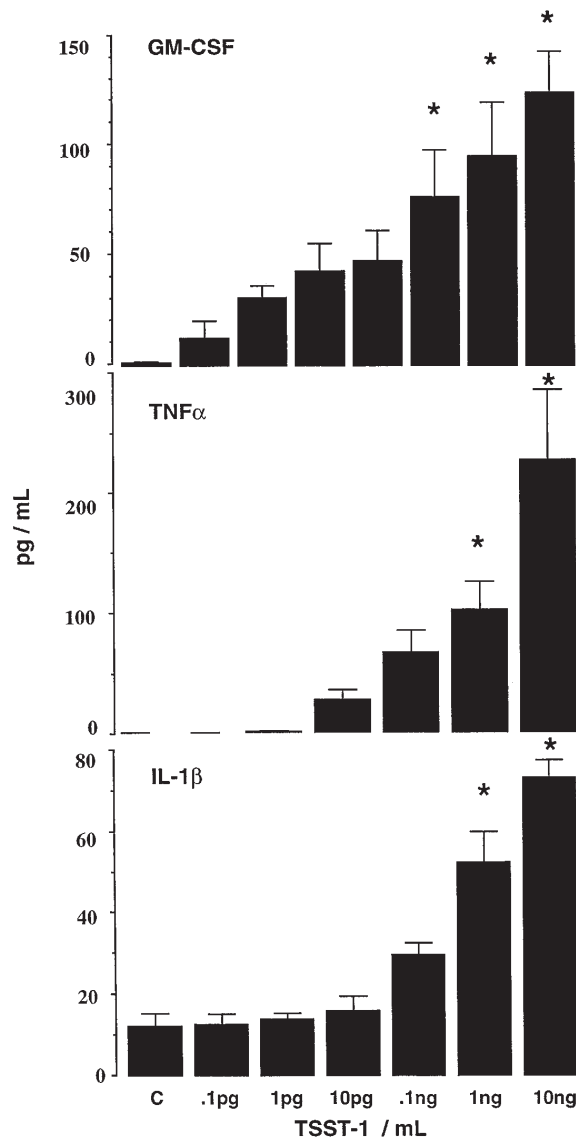


FIG 4. Cytokine production at 24 hours by TSST-1-treated monocytes. Cytokines were measured in supernatants harvested after 24 hours of incubation with various concentrations of TSST-1 as indicated. For each data set, asterisks note significant differences ($P < .05$) from control cells incubated in the absence of TSST-1, as determined by a univariate repeated-measures ANOVA and Tukey's multiple comparisons procedure. Data are represented as means $\pm$ SEM ($n = 4$ to 7 donors).

excess) singly and in combination were added with TSST-1 to freshly isolated normal monocytes. In no case was the incidence of apoptosis restored to control (unstimulated) levels, suggesting the possibility of ongoing autocrine or paracrine stimulation. Furthermore, inhibition of apoptosis was even more pronounced with the antibody additions, probably because of stimulation of the monocytes through the Fc receptor. Thus we resorted to coculturing of fresh normal monocytes in conditioned media harvested from previously isolated normal monocytes after 24 hours' incubation with TSST-1.

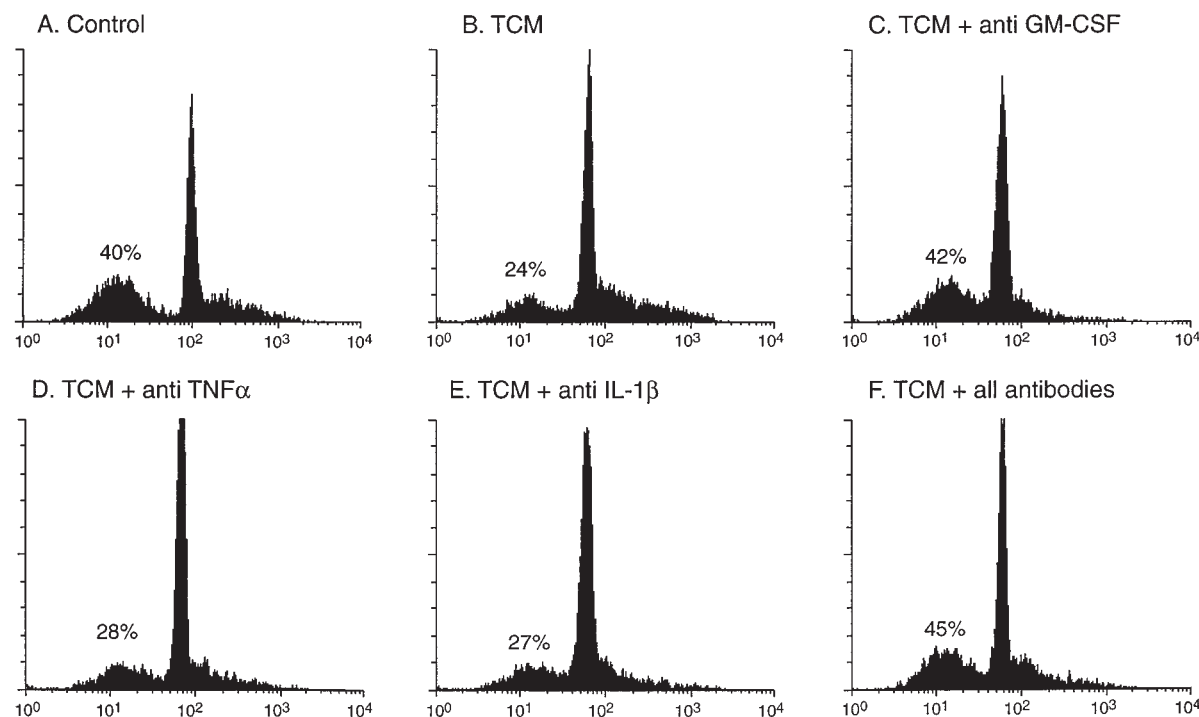


FIG 5. Histograms demonstrating apoptotic cells identified by hypodiploid DNA after culture of monocytes in conditioned media from previously isolated monocytes treated with TSST-1 (1 ng/mL) for 24 hours. All harvested supernatants were treated with neutralizing antibody to TSST-1, with or without neutralizing antibody to the indicated cytokine, and then added to fresh monocytes and incubated for 24 hours (see Methods section). Experiment is representative of 3 experiments.

TSST-1 was removed from the conditioned media by the addition of neutralizing antibody to TSST-1, and similarly the cytokines were variously removed with neutralizing antibodies. The antibodies were then removed with protein G, and the conditioned media was added to freshly isolated monocyte cultures. As shown in Fig 5, culturing of monocytes with prepared conditioned media from TSST-1-treated monocytes resulted in inhibition of apoptosis compared with untreated control cultures (24% vs 40%). Of note, the addition of neutralizing antibody to GM-CSF (Fig 5, C) inhibited this effect of the conditioned media by restoring the incidence of apoptosis (42%), whereas neutralizing antibody to TNF- α and IL-1 β showed only minor effects. Thus the data demonstrate that TSST-1 inhibits monocyte apoptosis by the production of prosurvival cytokines, the most important of which is GM-CSF.

DISCUSSION

Peripheral blood monocytes are a useful tool in investigating the inflammatory events of AD. Earlier reports have shown that AD monocytes produce increased amounts of PGE₂ and IL-10, cytokines that result in a shift to a T_{H2} pattern of cytokine production, with decreased IFN- γ production and enhanced IL-4 produc-

tion.¹⁴⁻¹⁶ Such findings in peripheral blood monocytes are reflected in lesional skin.¹⁶⁻¹⁸ Monocytes are also capable of producing a variety of cytokines, including GM-CSF, IL-1 β , and TNF- α , which have been shown to decrease monocyte apoptosis in vitro.^{4,12} Our previous work suggests that GM-CSF in particular plays a pivotal role in the inhibition of monocyte-macrophage apoptosis in chronic AD.⁴ The current data extend these observations and support the hypothesis that exotoxins produced by *S aureus* initiate production of GM-CSF, as well as the other prosurvival cytokines. As such, TSST-1, acting as a superantigen, may contribute to the chronicity of AD. Previous reports confirm TNF- α and IL-1 β production in response to superantigen stimulation of monocyte-macrophages but also potentially with stimulation of other HLA-DR-expressing cells found in AD skin lesions (Langerhans' cells and keratinocytes).¹⁸⁻²⁰ To our knowledge this is the first report of GM-CSF production by human monocytes in response to staphylococcal exotoxin,²¹ and it draws a parallel with that seen with superantigen activation of T cells.²²

In addition to prolonging monocyte survival, the presence of GM-CSF may be required to upregulate the low-affinity IgE receptor on monocyte-macrophages, which is necessary for IgE-mediated monocyte activation.²³ In turn, demonstrable increases in IL-1 β and TNF- α pro-

duction by monocytes have been found in response to IgE stimulation.²⁴ Finally, it has been shown that although IL-4 promotes monocyte apoptosis, this effect is abrogated by GM-CSF.^{4,25}

Such a scenario places both colonization of AD skin lesions with exotoxin-producing *S aureus* and IgE-mediated activation at the center for the prosurvival cytokine production necessary for the perpetuation of AD. Ongoing studies are directed at further dissection of the role of these inflammatory events. In conclusion, although the underlying mechanisms for initiation and perpetuation of AD inflammation remain elusive, our data suggest that staphylococcal exotoxins contribute to the chronicity of AD by stimulating GM-CSF and other prosurvival cytokines' production, resulting in both monocyte activation and inhibition of monocyte apoptosis.

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REFERENCES

1. Leung DYM. Atopic dermatitis: the skin as a window into the pathogenesis of chronic allergic diseases. *J Allergy Clin Immunol* 1995;96:302-18.
2. Mihm MC, Soter NA, Dvorak HF, Austen KF. The structure of normal skin and the morphology of atopic eczema. *J Invest Dermatol* 1976;67:305-12.
3. Leung DYM, Bhan AK, Schneeberger EE, Geha RS. Characterization of the mononuclear cell infiltrate in atopic dermatitis using monoclonal antibodies. *J Allergy Clin Immunol* 1983;71:47-56.
4. Bratton DL, Hamid Q, Boguniewicz M, Doherty DE, Kailey JM, Leung DYM. Granulocyte macrophage-colony stimulating factor contributes to enhanced monocyte survival in chronic atopic dermatitis. *J Clin Invest* 1995;95:211-8.
5. Hamid Q, Boguniewicz M, Leung DYM. Differential *in situ* cytokine gene expression in acute vs. chronic atopic dermatitis. *J Clin Invest* 1994;94:870-6.
6. Ying S, Meng Q, Taborda-Barata L, Kay AB. Association of apoptosis of neutrophils and eosinophils and their ingestion by macrophages with resolution of the allergen-induced cutaneous late-phase response in atopic human subjects. *Proc Assoc Am Physicians* 1997;109:42-50.
7. Simon H-U, Yousefi S, Schranz C, Schapowal A, Bachert C, Blaser K. Direct demonstration of delayed eosinophil apoptosis as a mechanism causing tissue eosinophilia. *J Immunol* 1997;158:3902-8.
8. Wedi B, Raap U, Lewrick H, Kapp A. Delayed eosinophil programmed cell death in vitro: a common feature of inhalant allergy and extrinsic and intrinsic atopic dermatitis. *J Allergy Clin Immunol* 1997;100:536-43.
9. Leyden JJ, Marples RR, Kligman AM. *Staphylococcus aureus* in the lesions of atopic dermatitis. *Br J Derm* 1974;90:525-30.
10. Leung DYM, Harbeck R, Bina P, Hanifin JM, Reiser RF, Sampson HA. Presence of IgE antibodies to staphylococcal exotoxins on the skin of patients with atopic dermatitis: evidence for a new group of allergens. *J Clin Invest* 1993;92:1374-80.
11. Marrack P, Kappler J. The Staphylococcal enterotoxins and their relatives. *Science* 1990;248:705-11.
12. Mangan DF, Welch GR, Wahl SM. Lipopolysaccharide, tumor necrosis factor- α , and IL-1 β prevent programmed cell death (apoptosis) in human peripheral blood monocytes. *J Immunol* 1991;146:1541-6.
13. Doherty DE, Haslett C, Tonnesen MG, Henson PM. Human monocyte adherence: a primary effect of chemotactic factors on the monocyte to stimulate adherence to human endothelium. *J Immunol* 1987;138:1762-71.
14. Chan SC, Li S-H, Hanifin JM. Increased interleukin-4 production by atopic mononuclear leukocytes correlates with increased cyclic adenosine monophosphate-phosphodiesterase activity and is reversible by phosphodiesterase inhibition. *J Invest Dermatol* 1993;100:681-4.
15. Chan S, Henderson J, Henderson WR Jr, Li S-H, Hanifin JM. Prostaglandin E₂ control of T cell cytokine production is functionally related to the reduced lymphocyte proliferation in atopic dermatitis. *J Allergy Clin Immunol* 1996;97:85-94.
16. Ohman JD, Hanifin JM, Nickoloff BJ, et al. Overexpression of IL-10 in atopic dermatitis: contrasting cytokine patterns with delayed-type hypersensitivity reactions. *J Immunol* 1995;154:1956-63.
17. Cooper KD. Atopic dermatitis: recent trends in pathogenesis and therapy. *J Invest Dermatol* 1994;102:128-37.
18. Herz U, Bunikowski R, Renz H. Role of T cells in atopic dermatitis. *Int Arch Allergy Immunol* 1998;115:179-90.
19. Trede NS, Geha RS, Chatila T. Transcriptional activation of IL-1 β and tumor necrosis factor- α genes by MHC Class II ligands. *J Immunol* 1991;146:310-5.
20. Tokura Y, Yagi J, O'Malley M, et al. Superantigenic staphylococcal exotoxins induce T-cell proliferation in the presence of Langerhans cells or class II-bearing keratinocytes and stimulate keratinocytes to produce T-cell-activating cytokines. *J Invest Dermatol* 1994;102:31-8.
21. Pastore S, Fanale-Belasio E, Albanesi C, Chinni LM, Giannetti A, Girolomoni G. Granulocyte macrophage colony-stimulating factor is overproduced by keratinocytes in atopic dermatitis. *J Clin Invest* 1997;99:3009-17.
22. Schols D, Jones D, Roncarolo M-G. Unique cytokine production profile of anergic human T cells in SCID-hu mice after staphylococcal enterotoxin B administration. *J Immunol* 1995;154:3204-12.
23. Matz J, Williams J, Rosenwasser J, Borish LC. Granulocyte macrophage colony stimulating factor stimulates macrophages to respond to IgE via the low affinity IgE receptor (CD23). *J Allergy Clin Immunol* 1994;93:650-7.
24. Borish L, Mascali JJ, Rosenwasser LJ. IgE-dependent cytokine production by human peripheral blood mononuclear phagocytes. *J Immunol* 1991;146:63-7.
25. Mangan DF, Wahl SM. Differential regulation of human monocyte programmed cell death (apoptosis) by chemotactic factors and pro-inflammatory cytokines. *J Immunol* 1991;147:3408-12.