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*J Immunol* 2000; 164:2897-2904; doi: 10.4049/jimmunol.164.6.2897

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TRAIL (Apo2 Ligand) and TWEAK (Apo3 Ligand) Mediate CD4\(^+\) T Cell Killing of Antigen-Presenting Macrophages\(^1\)

Mariana J. Kaplan,* Donna Ray,* Ru-Ran Mo,* Raymond L. Yung,* and Bruce C. Richardson\(^{2*\dagger}\)

The human marrow produces \(\sim 10^{10}\) monocytes daily, and this production must be balanced by a similar rate of destruction. Monocytes/macrophages can undergo apoptosis after activating CD4\(^+\) T cells, suggesting one mechanism that may contribute to macrophage homeostasis. Previous reports indicate that Fas-Fas ligand interactions are the principle molecules mediating this response. However, D10, an I\(\alpha\)\(^b\)-restricted cloned Th2 line, will similarly induce apoptosis in Ag-presenting macrophages, and D10 cells lack Fas ligand. To confirm that D10 cells kill macrophages through Fas-independent pathways, D10 cells were shown to kill MRL lpr/lpr (I\(\alpha\)\(^b\)) macrophages in an Ag-dependent fashion, indicating additional mechanisms. Recent reports demonstrate that TNF-related apoptosis-inducing ligand (TRAIL), interacting with Apo2, and TNF-like weak inducer of apoptosis (TWEAK), interacting with Apo3, will induce apoptosis in some cells. Using Abs to TRAIL and an Apo3-IgG Fc fusion protein, we demonstrated that D10 cells express both TRAIL and TWEAK. The Apo3 fusion protein, but not human IgG, inhibited D10-induced macrophage apoptosis, as did anti-TRAIL. Further studies demonstrated that AE7, a cloned Th1 line, and splenic T cells express TWEAK, TRAIL, and Fas ligand, and inhibiting these molecules also inhibited macrophage killing. These results indicate that D10 cells induce macrophage apoptosis through TRAIL- and TWEAK-dependent pathways. Because normal T cells also express these molecules, these results support the concept that T cells have multiple pathways by which to induce macrophage apoptosis. These pathways may be important in immune processes such as macrophage homeostasis as well as in down-regulation of immune responses and elimination of macrophages infected with intracellular organisms. *The Journal of Immunology, 2000, 164: 2897–2904.

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Received for publication June 29, 1999. Accepted for publication January 3, 2000.

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\(^{*}\) This work was supported by U.S. Public Health Service Grants AR42525, AG014783, and AI42753; a Veterans Affairs Merit Review grant; and an award from Amgen through the Life Sciences Foundation (to M.J.K.).

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\(^{\dagger}\) Abbreviations used in this paper: M\(\phi\), macrophage; FasL, Fas ligand; CD40L, CD40 ligand; DR, death receptor; TRAIL, TNF-related apoptosis-inducing ligand; TWEAK, TNF-like weak inducer of apoptosis.

Materials and Methods

**Animals, cells, and cell lines**

Six- to eight-week-old female AKR, MRL \(^{+/+}\), MRL lpr/lpr, and B10.A mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Peritoneal M\(\phi\) were elicited by i.p. injection of thioglycolate (Becton Dickinson, Cockeysville, MD), and the cells were recovered 3 days later as previously described (9). D10,G4.1 (D10), a cloned Th2, H-\(^{2}\) restricted, conalbumin-reactive Th2 line was obtained from American Type Culture Collection.
Collection (Manassas, VA) and was maintained by repeated restimulation with irradiated splenocytes and conalbumin (Sigma, St. Louis, MO) and the regular addition of IL-2 as previously described (10). Because of a report that the D10.G4.1 cell line may contain an autoreactive subset (20), the D10 cells were subcloned by limiting dilution at <0.2 cells/well, and a nonautoreactive subclone was selected for use in these studies. Cells were studied 6 days after challenge to avoid interference by irradiated stimulator cells (10). Where indicated, the cells were stimulated with 1 ng/ml PMA and 1 μg/ml ionomycin (Sigma). AE7 cells were obtained from Dr. Ronald Schlossman (21) and cultured by repeated stimulation with syngeneic (B10.A) irradiated splenocytes and Ag (100 μg/ml pigeon cytochrome-c from Sigma), in Clicks medium supplemented with penicillin/streptomycin and 1-glutamine, 10% FBS, 2 mM-l-glutamine, 50 μM 2-ME, and IL-2 (9). CTLL cells were obtained from American Type Culture Collection and maintained according to the instructions provided. Where indicated, AKR and B10.A splenocytes were stimulated by culture for 18 h in flat-bottom 96-well microtiter plates (Costar, Cambridge, MA) coated with 10 μg/ml anti-CD3 (2C11, PharMingen, San Diego CA). Alternatively, cells were stimulated with 2 μg/ml of Con A (Sigma). AKR and B10.A splenocytes were obtained by gently mincing the spleens through a steel screen, and the collected splenocytes were washed twice with PBS/5% FBS, then purified by density gradient centrifugation. Where indicated, CD4+ cells were enriched by treating the splenocytes with anti-CD8 and complement (9). This typically gave ~40% CD4+ and 0% CD8+ cells by flow cytometric analysis. Cell viability was measured with propidium iodide staining and was >95% in each cell preparation used. Splenocytes were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (pH 7.2), and 2 mM glutamine at 37°C with 5% CO2 in a humidified atmosphere.

Abs and reagents

Anti-murine CD40, anti-murine CD40 ligand (CD40L), anti-murine FasL, anti-murine CD3-FITC were obtained from PharMingen; recombinant TNF was obtained from R&D Systems (Minneapolis, MN), and an inhibitory anti-murine TNF was a gift from Dr. Steven Kunkel. Anti-IL-4 was obtained from Endogene (Cambridge, MA), and rIL-4 was purchased from R&D Systems. A murine TRAMP (Apo3) IgG1 Fe fusion protein (anti-TWEAK) was a gift from Immunex (Seattle, WA). The S-H4o rabbit anti-TRAIL antibody was a gift from Dr. Peter Krämer, M3-IgG1-FITC, and anti-human rabbit-FITC, and rat anti-human IgG-FITC were obtained from Coulter (Miami, FL). Anti-FasL was purchased from PharMingen. PHA was obtained from Murex Diagnostics (Norcross, GA).

Flow cytometric analysis

For D10 and AE7 cells, one million cells were incubated for 30 min at 4°C with anti-TWEAK or anti-TRAIL, diluted to 5 μg/ml in PBS, washed three times, then incubated for 30 min at 4°C with FITC-conjugated Abs to human or rabbit IgG, respectively. Cells were washed three times, fixed in formaldehyde, and analyzed in a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA) using previously described protocols (9, 10). FasL expression was similarly detected using anti-FasL and FITC-conjugated anti-hamster IgG (PharMingen). For splenocytes, the stained cells were washed three times, then incubated with anti-CD3-PE (PharMingen) and analyzed by two-color flow cytometry using published protocols (9, 10). Peritoneal macrophages were similarly stained, then analyzed by gating on the macrophage population as assessed by light scatter. This population contained <1% CD3+ cells as detected by staining with anti-CD3-PE.

Electron microscopy

Transmission electron microscopy of T cells and Mφ was performed as previously described (8).

Cytotoxicity assays

D10 Mφ killing assays were performed as previously described (9, 10). Briefly, thioglycolate-elicited peritoneal Mφ were radiolabeled with 51Cr (New England Nuclear, Boston, MA) for 1 h. The cells were washed, then 5,000 labeled Mφ were cultured with 125,000 D10 cells and 100 μg/ml conalbumin in a total volume of 200 μl of medium lacking IL-2, using round-bottom microtiter plates. Where indicated, Abs or fusion proteins were added to the wells. Eighteen hours later chromium release was measured using a scintillation spectrometer. AE7 killing assays were performed similarly except that an E:T cell ratio of 10:1 was used, and the Ag was pigeon cytochrome c, added at 100 μg/ml. Splenocyte killing assays were performed by stimulating CD4-enriched splenocytes with Con A for 72 h, then the cells were washed three times and cultured with 51Cr-labeled allogeneic peritoneal Mφ at an E:T cell ratio of 25:1 in medium containing 0.1 M α-mercapto-3-mannoside. Cytotoxicity was measured 18 h later as described for D10 and AE7 cells.

Proliferation assays

Proliferation assays were performed as previously described (9, 10). Briefly, 125,000 D10 cells were cultured with 5,000 irradiated AKR splenocytes and 100 μg/ml conalbumin in a total volume of 200 μL again using IL-2-free medium. Where indicated anti-TWEAK, anti-TRAIL, or a control Ig was added at concentrations identical with those used for the cytotoxicity assays. After 72 h tritiated thymidine (New England Nuclear) was added, and 4 h later cells were harvested, and radioactivity was measured using a scintillation spectrometer.

Northern analysis

Northern analysis was performed as previously described (22, 23). In brief, mRNA was isolated from unstimulated D10 cells, from D10 cells stimulated with PMA and ionomycin, from CTLL cells, or from AKR splenocytes that had been treated with PHA or PMA and ionomycin. The RNA was fractionated by electrophoresis, transferred to nylon membrane, hybridized with a32P-radioabeled probe, and developed as an autoradiogram. The probes used included FasL, amplified by RT-PCR from CTLL cells using primers CTTCGGCTGTTGGTGCATTGC and TCTGTGCCTGACATCTCGCTCC and previously described protocols (22, 23). A perforin probe was similarly amplified using primers CGCACTTATATCACGCTGTGGACCTGCTG and GTGGGCAGCAGTCTCCTGTTGT GTGACCTTTG.

Statistical analysis

The difference between means was tested using Student’s t test.

Results

Fas is not required for D10-induced Mφ apoptosis

Initial experiments confirmed that D10 cells kill Mφ by apoptosis. Electron microscopy was used to distinguish apoptosis from necrosis (8). Fig. 1a shows two normal, thioglycolate-stimulated, AKR peritoneal Mφ cultured alone for 18 h. The Mφ have prominent vacuolar inclusions due to the thioglycolate. Fig. 1b shows a T cell adherent to a Mφ, with what appears to be early nuclear condensation, in cultures of AKR Mφ, D10 cells, and conalbumin. Fig. 1, c and d, shows representative electron micrographs of AKR Mφ similarly cultured with D10 cells and conalbumin, showing typical nuclear condensation with preservation of cytoplasmic structures. These changes are typical of apoptosis. To exclude the possibility that the apoptotic Mφ observed represent spontaneous apoptosis of the Mφ, low power electron micrographs of the entire cell pellet were made, and the percentage of apoptotic Mφ was determined by visual inspection. For each determination, five fields were examined, and the percentage of apoptotic Mφ in 100 total Mφ was determined. In cultures of Mφ without D10 cells, 8 ± 1% of the Mφ were apoptotic, while cultures of Mφ with D10 cells and Ag contained 40 ± 3% apoptotic cells (p < 0.001).

T lymphocytes use FasL and perforin in target cell killing (13, 14), but D10 cells reportedly lack FasL and perforin expression (16, 18, 19). Because D10 cells clearly killed Mφ, evidence for perforin and FasL expression was sought to exclude the possibility that this D10 subclone differed from those previously reported. Northern analysis was used to demonstrate that splenocytes treated with PMA and ionomycin express FasL mRNA, but that D10 cells and D10 cells similarly treated with PHA do not (not shown). Similarly, flow cytometric analysis demonstrated that stimulated splenocytes expressed FasL (typically 15–25% positive; see Fig. 7), while D10 cells did not (MCF 0.325 vs 0.431, FasL vs isotype control staining of D10 cells). In similar experiments using Northern analysis and CTLL cells as positive controls, we were also unable to induce perforin expression in D10 cells (not shown). These results confirm previous studies with D10 cells (16, 18, 19).
To further exclude a role for Fas-FasL interactions in D10-mediated Mφ killing, we used MRL lpr/lpr peritoneal Mφ. This strain is derived from the MRL lpr/lpr strain, but has a retroviral insertion in the Fas gene, resulting in a nonfunctional transcript (24). Importantly, the MRL strains are also H-2k, similar to the D10 cells (25). Varying numbers of D10 cells were cultured with 5,000 51 Cr-labeled AKR, MRL lpr/lpr, or MRL lpr/lpr Mφ (Fig. 2). The D10 cells lysed AKR and MRL lpr/lpr Mφ equally well. Lysis of the MRL lpr/lpr Mφ was somewhat less efficient, but maximum lysis was as great as that seen with AKR and lpr/lpr target cells. This argues that Mφ Fas is not required for D10-mediated Mφ killing.

Other mechanisms of Mφ apoptosis

These results prompted studies of other mechanisms proposed for apoptosis. Because D10 cells secrete IL-4 (10), and this cytokine has been implicated in inducing Mφ apoptosis (5), 0.01–1.0 ng/ml IL-4 was added to AKR Mφ cultures for 18 h. No cytotoxicity was observed (1 ± 1.2% chromium release above background, using 1 ng/ml IL-4). In addition, no significant inhibition was seen when 0.25–25 μg/ml neutralizing anti-IL-4 was added to cultures of D10 cells, Mφ, and Ag (maximum specific killing, 41 ± 1.8% in the presence of 25 μg/ml anti-IL-4 vs 56 ± 8% killing in controls (p > 0.05), with less inhibition at lower concentrations). This argues against a prominent role for IL-4 in this response. In B cells, CD40-CD40L interactions protect from apoptosis (26), so it was possible that a lack of CD40L expression on D10 cells could increase apoptosis. However, flow cytometric analysis demonstrated that D10 expressed CD40L (MCF 5.45 vs 0.56 anti-CD40L vs control, 79.4% positive).

Role of TWEAK and TRAIL in D10-induced Mφ apoptosis

More recently, two newly identified molecules have been implicated in triggering apoptosis. These include TRAIL, which interacts with molecules including Apo2 (27–31), and TWEAK, which...

FIGURE 1. Electron microscopy of Mφ killed by D10 with Ag. A, AKR peritoneal Mφ cultured alone (magnification, ×6200). B, D10 cell binding to AKR peritoneal Mφ in the presence of Ag (magnification, ×6900). C and D, Apoptotic Mφ detected in cultures of D10 cells and Ag (magnification, ×5450).

FIGURE 2. D10 killing of lpr Mφ. The indicated numbers of D10 cells were cultured with 5000 51Cr-labeled AKR (solid line), MRL lpr/lpr (dotted line), or MRL lpr/lpr (dashed line) peritoneal Mφ, and the percent cytotoxicity was measured 18 h later. Results represent the mean ± SEM of quadruplicate determinations.
interacts with Apo3 (32, 33). TRAIL was detected using a rabbit anti-mouse TRAIL antisera, while TWEAK was detected using a TRAMP (Apo3)/human IgG1 Fc fusion protein, followed by goat anti-human IgG-FITC. The control consisted of 5 μg of purified human IgG, followed by anti-human Ig-FITC.

We next asked whether D10-induced Mφ apoptosis was due to TWEAK and/or TRAIL interactions. Graded amounts of the anti-TRAIL or the Apo3 fusion protein (anti-TWEAK) were added to cultures of AKR peritoneal Mφ, D10 cells, and conalbumin. Fig. 4 demonstrates that both inhibited killing, but anti-TRAIL was somewhat more potent under the conditions used. Concentrations of anti-FasL equal to the highest tested concentrations of anti-TWEAK had no significant inhibitory effect (24 ± 14.8% inhibition, mean ± SEM of three independent experiments, each performed in quadruplicate). The possibility that anti-TWEAK or anti-TRAIL inhibited D10 killing through a toxic effect on the D10 cells was excluded by propidium iodide staining. Control D10 cells were 21% propidium iodide positive, while cells incubated with anti-TWEAK and anti-TRAIL ranged from 19–23% propidium iodide positive. Since we had previously reported that inhibiting T cell activation with mAb to molecules such as LFA-1 or class II MHC also prevents Mφ killing (8), it was important to exclude the
possibility that the anti-TWEAK and TRAIL reagents were inhibiting T cell activation. Using conditions similar to the cytotoxicity assay, anti-TWEAK had no effect on D10 activation, while anti-TRAIL had an inhibitory effect that was smaller in magnitude than the effect on killing (Fig. 4b).

Because both reagents inhibited killing completely, it was possible that the reagents were interfering with each other through steric hindrance. However, when D10 cells were cultured with saturating amounts of anti-TWEAK, then stained for TRAIL expression, no competition was observed (Fig. 5). This argues against steric hindrance.

Comparison of TWEAK, TRAIL, and FasL in T cell-induced Mφ apoptosis

These results indicate that TWEAK and TRAIL can participate in D10-induced Mφ apoptosis. However, FasL can also mediate Mφ apoptosis (16), so it was of interest to compare the relative contributions of these three molecules in macrophage killing by CD4+ T cells. AE7 cells were used for these studies, because they have been reported to delete macrophages through a Fas-dependent pathway (16). Fig. 6A compares the relative expressions of FasL, TWEAK, and TRAIL on AE7 cells. All three molecules are expressed. Fig. 6B compares the inhibitory effects of Abs to each of these molecules. As was seen for D10 cells, both anti-TWEAK and anti-TRAIL inhibit Mφ killing. In addition, anti-FasL inhibits at concentrations similar to those of anti-TWEAK.

The roles of TWEAK, TRAIL, and FasL in Mφ apoptosis were also compared using splenic T cells. Splenocytes were stimulated for 18 h with anti-CD3 immobilized on flat-bottom tissue culture plates, then stained with anti-CD3-PE and anti-TWEAK, anti-TRAIL, or anti-FasL as before. Fig. 7, A–C, shows that TWEAK, TRAIL, and FasL are expressed on unstimulated CD3+ cells, and expression increases following stimulation. The effects of anti-TWEAK, anti-TRAIL, and anti-FasL on splenocyte killing of allogeneic macrophages were then tested. Con A-stimulated, CD8-depleted splenocytes from B10.A mice (H-2a) were cultured with 51Cr-labeled AKR (H-2k) peritoneal Mφ and the same concentrations of anti-TRAIL, anti-TWEAK, and anti-FasL as those given in Fig. 6. Again, all three proteins inhibited Mφ killing (Fig. 7D), supporting a role for these molecules in inducing Mφ apoptosis.
Expression of TRAIL, TWEAK, and FasL on peritoneal Mφ

Finally, the possibility that Mφ express TWEAK and TRAIL was explored. AKR peritoneal Mφ were stained with anti-TRAIL, anti-TWEAK, and anti-FasL as before and were analyzed by flow cytometry. TRAIL was expressed on 24.9 ± 8.2% of the Mφ, while TWEAK was expressed on 63.6 ± 3.8% and FasL on 14.8 ± 4.0% (mean ± SEM of three independent experiments relative to negatively staining controls).

Discussion

These studies identify two previously unreported mechanisms that participate in the Mφ death response, which commonly occurs after CD4+ T cell clones are stimulated by Ag-presenting Mφ. Using human cells, we reported that optimal induction of apoptosis requires physical contact between T cells and Mφ and could not be induced by soluble mediators released from the T cells (8), although we were unable to exclude the possibility that locally high concentrations of a secreted molecule acted at close range. Additional studies demonstrated that cross-linking specific Mφ surface molecules, including class I and II MHC, ICAM-1 (CD54), LFA-1 (CD11a/CD18), and LFA-3 (CD58), alone or in combination, was similarly ineffective (8). Others have since reported that Mφ killing by Th1 T cell clones is largely mediated through Fas/FasL interactions (16). However, D10 cells, which lack FasL expression (16, 18, 19), are also capable of killing syngeneic Ag-presenting Mφ (10, 17). Similarly, others have reported that polyclonal CTLs deficient in both FasL and perforin have residual cytolytic activity (34), and that FasL and perforin/granzyme mechanisms account for ~40% of the total cytotoxicity of CD4+ CTLs (35). TNF has been implicated in inducing apoptosis in some systems and inhibiting it in others (35–37). However, neither addition of TNF nor inhibition with neutralizing Abs had any effect on D10 killing of Mφ (our unpublished observations). Together, these observations indicate the existence of additional cytotoxic mechanisms.

The present studies demonstrate that D10 cells as well as AE7 and freshly isolated murine T cells express TWEAK and TRAIL, and that these molecules participate in Mφ apoptosis. TRAIL is a TNF family member that activates apoptosis and binds to death receptor 4 (DR4) and DR5 (27–31). DR5 is also referred to as Apo2 (38). TRAIL mRNA is also expressed in PBMC, spleen, thymus, prostate, ovary, small intestine, colon, and placenta, but not in brain, liver, or testis (27). TRAIL has been found to be involved in the activation-induced death of Jurkat and human peripheral blood T cells (39) and to induce apoptosis in Fas ligand-resistant melanoma
cells (28). TRAIL also mediates CD4 T cell killing of target cancer cells (35). Others have suggested that TRAIL primarily participates in the killing of transformed cells (31). Our results suggest a homeostatic role for this molecule as well.

TWEAK is a recently described, 249-amino acid, type II transmembrane protein, whose extracellular sequence shows highest identity to that of TNF. It is expressed on a majority of adult and fetal tissues (32, 33). TWEAK binds Apo3, which is also referred to as DR3, WSL-1, TRAMP, or LARD (33, 40). Significantly, Apo3 expression appears to be restricted to lymphocyte-rich tissues such as PBMC and spleen (40–44). Soluble TWEAK has been reported to induce apoptosis and NF-κB activation in human cell lines (41), and caspase inhibitors can block apoptosis induction by TWEAK (32). TWEAK has overlapping signaling functions with TNF. However, TWEAK is generally less effective in inducing apoptosis than other molecules, giving rise to its name, TNF-like weak inducer of apoptosis (32). Proteins binding both molecules appear to inhibit the apoptotic response, and at high concentrations both completely inhibit this response in D10 cells. Trivial explanations, such as a toxic effect, were excluded. The inhibition is not due to blocking T cell activation, because concentrations of the inhibitors that blocked killing had lesser effects on T cell proliferative responses. Similar inhibition experiments performed using AE7 cells, a cloned Th1 line that expresses FasL, TWEAK, and TRAIL, as well as CD4-enriched splenocytes demonstrated that proteins binding all three molecules similarly inhibit killing by >80%. These results suggest that TWEAK and TRAIL are excellent candidates for the molecules mediating D10 killing of Ag-presenting syngeneic MΦ. The reason for nearly complete inhibition by each inhibitor is uncertain, but possibilities include a steric inhibition not detected by the competitive inhibition approach used, or that signals generated by binding any one of these molecules on the T cell surface inhibit the ability of the other molecules to deliver an apoptotic signal. Future studies will examine these possibilities. These results also raise the possibility that TWEAK and TRAIL may be responsible for the residual cytotoxic activity observed in other CD4+ CTLs (34, 35).

These studies may be important for MΦ homeostasis. As noted previously (8), the human bone marrow generates 7 × 10⁸ monocytes/kg body weight/h (1, 2). Because MΦ can be long-lived, mechanisms for their elimination are necessary. It is also possible that killing of Ag-presenting MΦ by CD4+ T cells serves to down-regulate immune responses by eliminating the APC. This would suggest that a constant supply of both Ag and APC is required to maintain an immune response. Finally, MΦ killing may be necessary to eliminate cells infected with intracellular parasites. It is relevant to note that mice lacking both perforin and FasL develop an autoimmune disease with largely lymphocytic mononuclear cell infiltration of the pancreas and uterus (45). This suggests that TWEAK and TRAIL are insufficient to compensate for loss of perforin and FasL in the homeostasis of lymphocytes. However, analysis of the lymphoid and other tissues of these mice does not demonstrate an increase in monocytes/macrophages, further suggesting that TWEAK and TRAIL are able to compensate for macrophage homeostasis.

These results may also be important for the development of autoantibodies in at least one animal model of lupus and possibly idiopathic human systemic lupus erythematosus. D10 cells overexpressing LFA-1 become autoreactive and respond to syngeneic MΦ without conalbumin, promiscuously killing MΦ in vitro (17). The same cells induce anti-DNA Abs in vivo (17), suggesting that this killing might contribute to the autoantibody response by providing a source of antigenic DNA. In support of this, patients with idiopathic systemic lupus erythematosus have increased numbers of circulating apoptotic monocytes (11), which may serve a similar function. Now that candidates for the responsible molecules have been identified, it is possible to test whether inhibiting D10-mediated MΦ killing in vivo prevents the development of autoantibodies.

Acknowledgments

We thank Drs. Peter Krammer, Steven Kunkel, and Ronald Schwartz and the personnel at Immunex for providing crucial reagents; Robin Kunkel for the electron microscopy; Dr. Laura Beretta-Hanash for her careful review of this manuscript; and Cindy Harper for expert secretarial assistance.

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