The Specific Recognition by Macrophages of CD8+,CD45RO+ T Cells Undergoing Apoptosis: A Mechanism for T Cell Clearance during Resolution of Viral Infections

By Arne N. Akbar,* John Savill,‡ Wendy Gombert,*
Margarita Bofill,* Nicola J. Borthwick,* Fiona Whitelaw,§
Jane Grundy,* George Janossy,* and Michael Salmon‖

From the *Department of Clinical Immunology, Royal Free Hospital School of Medicine,
London NW3 2QG; the ‡Division of Renal and Inflammatory Diseases, Department of
Medicine, University Hospital, Queens Medical Centre, Nottingham, NG7 2UH; the §Infectious
Diseases Unit, Coppett's Wood Hospital, London N10 1JN; and the ‖Department of
Rheumatology, University of Birmingham Medical School, Birmingham, B15 2TJ, United
Kingdom

Summary

During viral infections, CD8+,CD45RO+ T populations expand. These primed cells express abundant levels of cytoplasmic granules that contain perforin and TIA-1. Recent work has suggested that the majority of this CD8+ population downregulates Bcl-2 protein expression and is destined to undergo apoptosis. In this study we have investigated the elimination of these apoptotic CD8+ T cells by both human monocyte–derived and murine bone marrow macrophages. We have found that these phagocytes recognize and ingest both apoptotic CD8+ and CD4+ T cells using an $\alpha_v\beta_3$ (vitronectin receptor)/CD36/thrombospondin recognition system, with the same receptors being used in the recognition of apoptotic neutrophils. These data provide new evidence for a mechanism that enables the clearance of greatly increased populations of CD8+ effector cells which are found during viral infections. This enables cellular homeostasis to occur in the host upon resolution of viral diseases in vivo.

S elected T cell populations proliferate during viral infections in vivo which can result in a 20–30-fold increase in the frequency of virus-specific cytotoxic T lymphocyte precursor cells (1). This expansion can manifest itself as a CD8+ lymphocytosis accompanied by lymphadenopathy which is largely accounted for by a selective increase of the primed CD8+,CD45RO+ and to a lesser extent CD4+, CD45RO+ populations (2). After resolution of acute infections by viruses such as EBV, Varicella Zoster, and influenza, both the activated CD8+ and CD4+ T cell numbers return to normal levels (1–3). This appears to be due to apoptosis of the expanded populations, a process that is closely correlated with the downregulation of the bcl-2 gene product (4, 5). However, the mechanisms by which apoptotic T cells may be removed in vivo have received little attention. If disintegration of apoptotic CD8+ T cells occurred, the cytotoxic granules would be released into the extracellular compartment of tissues. Although not formally proven as yet, it is possible that this may lead to the nonspecific damage of normal healthy/uninfected cells (6).

The usual fate of cells that undergo apoptosis in vivo is rapid recognition and phagocytosis by macrophages (7, 8).

Indeed, during resolution of inflammatory responses it has been shown that neutrophils are eliminated by apoptosis and taken up by macrophages while still intact without release of their potentially toxic contents (7). In this study we demonstrate that apoptotic CD8+,CD45RO+ cells from patients with acute viral infections can be recognized and ingested by human monocyte–derived macrophages in vitro and that these processes may also occur in vivo.

Materials and Methods

Patients and Control Samples. Heparinized peripheral blood was obtained from normal individuals and patients with infectious mononucleosis within 10 d of the onset of symptoms. Lymph node biopsies were obtained from HIV-1–infected individuals (4).

Antibodies Used in the Study. The CD45RA (SN130; IgG₁), CD45RO (UCHL1; IgG₂ₐ) CD4 (RFT4; IgG₁), CD8 (RFT8; IgG₁ or IgM), and HLA-DR mabs (RFDR1; IgM) have been described previously (4). Macrophages in lymph node sections were identified using a CD68 antibody (EBM11; Dako Ltd., High Wycombe, UK). An antibody against perforin (PFP; IgG₂ᵦ), was kindly provided by Professor Bo Dupont (Laboratory of Human Immunogenetics, Sloan-Kettering Cancer Center, New York; ref.

9). Another cytotoxic granule protein was identified by the antibody TIA-1 (IgG₁; Coulter Clone, Luton, UK). P140, an IgG₂ₐ antibody that recognizes the GpIIb–IIIa complex, and Smφ (CD36), an IgM antibody, were gifts from Dr. Nancy Hogg (Imperial Cancer Research Fund, London, UK). C6.7, a thrombospondin-specific mAb of IgG₁ subclass was a gift from Dr. Vishva Dixit (University of Michigan, Ann Arbor, MI), while 23C6 mAb (IgG₁), which reacts with the αᵥβ₃ integrin, was a gift from Dr. Mike Horton (St. Bartholomew's Hospital, London, UK). These antibodies have been described previously (10).

Lymphocyte Activation and Generation of Apoptotic CD8⁺ and CD4⁺ T Cells. T cell populations were isolated and IL-2–dependent lines were generated as described (4, 5). To induce apoptosis, IL-2 was withdrawn and the cells were cultured for a further 48 h in the absence of this cytokine. To obtain apoptotic CD8⁺ T cells from patients with EBV infections, freshly harvested cells were cultured for 24 h without exogenous stimuli (4).

Cell Staining. T cells were analyzed by two- and three-color immunofluorescence and analyzed on the FACScan® (Becton Dick-

inson Ltd., Oxford, UK) or Cytoron Absolute (ORTHO Diagnostics, High Wycombe, UK). Cytocentrifuge preparations and histological sections were stained as described previously (4).

To examine apoptotic cells in tissue sections CD68 antibody (EMB11) was used to identify macrophages followed by propidium iodide (PI) staining. To investigate the TIA-1 reactivity of macrophage-ingested apoptotic CD8⁺ T cells, cytocentrifuge preparations were fixed in acetone incubated with TIA-1 antibody followed by a goat anti–mouse Ig antiserum second layer antibody labeled with horseradish peroxidase (Dako Ltd.). The slides were developed in a solution of diamino-benzidine with 1% H₂O₂, and the reaction was enhanced with a solution of cobalt chloride followed by counterstaining with May-Grunewald-Giemsa.

Macrophage Recognition and Blocking Studies. The phagocytic assay employed has been described previously (8, 10). Inhibitors included mAbs Arg-Gly-Asp-Ser (RGDS) and control Arg-Gly-Glu-Ser (RGES) peptides, phospho-L-serine, and control phosphatidylserine (PS)-derivative phospho-D-serine, and glycerophosphorylserine, all obtained from Sigma Chemical Co. (Poole, UK).
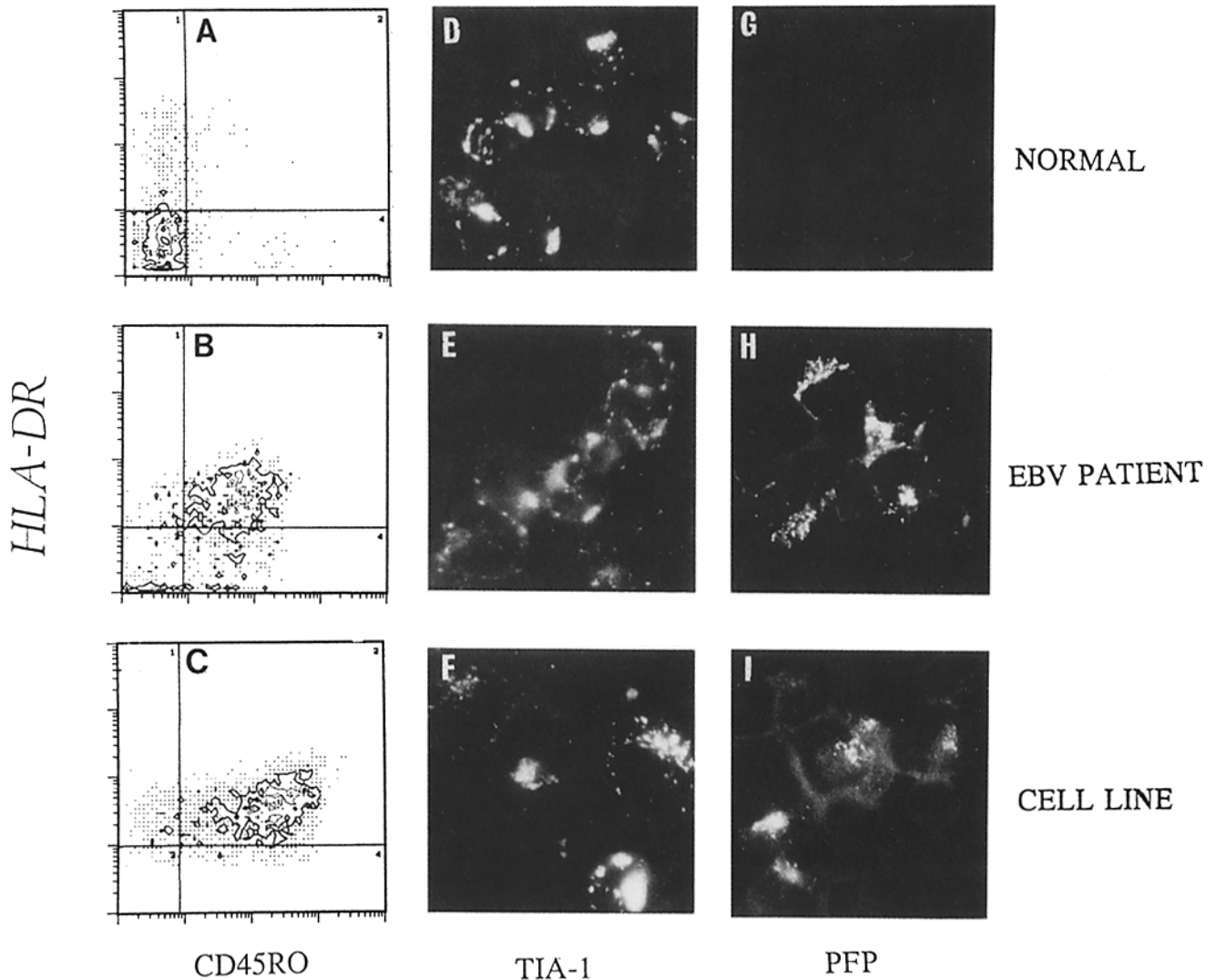

Figure 1. The expression of TIA-1 and perforin (PFP) by CD8⁺ T cell populations. Three-color immunofluorescence was performed on T cells isolated from normal individuals (*A*), patients with acute EBV infection (*B*), and IL-2–dependent CD8⁺ T cell line established from a normal healthy individual (*C*) using CD8, CD45RO, and HLA-DR antibodies. The CD8⁺ bright population was gated, and the expression of CD45RO and HLA-DR was determined on 10,000 cells. TIA-1 and perforin expression was determined in cytocentrifuge preparations of the CD8⁺ T cells from the normal individual (*D* and *G*), the EBV-infected patient (*E* and *H*), and the IL-2–dependent CD8⁺ line (*F* and *I*); 300–500 cells were counted per slide.

Results

The Expression of TIA-1 and Perforin by CD8+ T Cells.
The CD8+ T cell subset of normal individuals contained <4% of cells that coexpressed both HLA-DR and CD45RO (Fig. 1 A). In EBV-infected patients, 50–80% of the expanded CD8+ pool coexpressed HLA-DR and CD45RO, indicating recent activation (Fig. 1 B). IL-2–dependent CD8+ cell lines showed a comparable CD8+,CD45RO+,HLA-DR+ phenotype to the CD8+ T cell populations from EBV-infected patients (Fig. 1 C). When CD8+,CD45RO+ T cells from healthy individuals was purified, 40 ± 12% (mean ± SEM%) contained TIA-1 (Fig. 1 D) whereas 9.4 ± 3.5% contained low levels of perforin (n = 8; representative result shown in Fig. 1 G). In EBV-infected patients, 82.1 ± 6.5% of the CD8+,CD45RO+ T cells expressed TIA-1 (Fig. 1 E) whereas 60.8 ± 8.7% of cells brightly stained for perforin (n = 8; Fig. 1 H), a significant increase over the values seen in healthy individuals (p <0.001). The CD8+ IL-2–dependent line showed comparable TIA-1 and perforin reactivity to the CD8+,CD45RO+ cells from EBV-infected individuals.

The Preferential Death of CD8+,CD45RO+ T Cells from EBV Patients In Vitro. T cells were isolated from patients with EBV infections and cultured in medium alone with no added growth factors for 3 d (Fig. 2). The cells were analyzed by three-color immunofluorescence for CD4, CD45RA, and CD45RO as well as for CD8, CD45RA, and CD45RO by flow cytometry, in parallel with the absolute cell counts determined on the Cytoron. CD8+,CD45RO+ T cells were
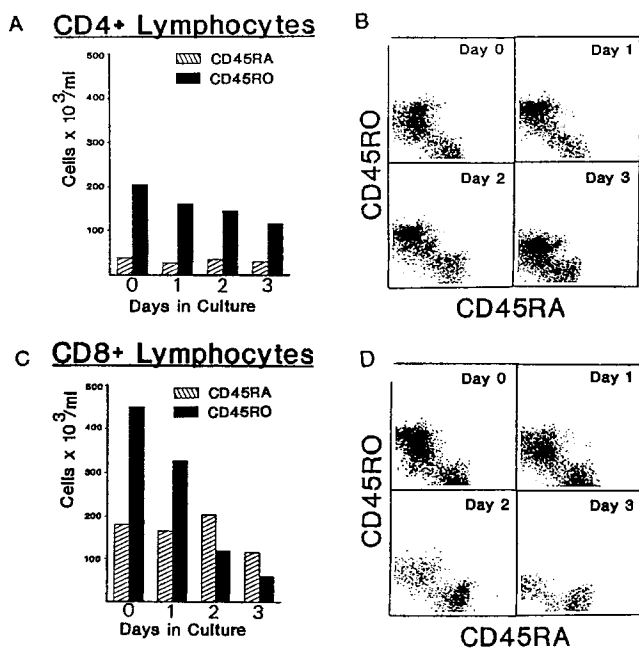

Figure 2. The preferential death of the CD8+,CD45RO+ population from EBV patients on culture without any exogenous stimuli in vitro. Peripheral blood mononuclear cells from a patient with EBV infection were cultured in medium with 10% FCS but without any other stimuli. Cells were harvested at the times shown and investigated for changes in viable CD4+ (A and B) and CD8+ (C and D) lymphocyte subsets by three-color immunofluorescence using CD4 or CD8 together with CD45RA and CD45RO antibodies. The absolute numbers of CD4+ (A) and CD8+ (C) subsets that were present at various times after culture were also determined using a Cytoron Absolute flow cytometer.

shown to be preferentially susceptible to death as their numbers selectively decreased during the incubation period (Fig. 2).

The Phagocytosis of Apoptotic T Cells by Macrophages. Of the apoptotic CD8+ T cells, >98% retained their membrane integrity when used in the phagocytic assays as shown by trypan blue dye exclusion. Freshly isolated nonapoptotic CD8+ T cells and IL-2–dependent CD8+ lines cultured in optimal concentrations of IL-2 were not susceptible to phagocytosis by human monocyte–derived macrophages. On removal of IL-2, however, 25–40% of the CD8+ T cells appeared apoptotic after 24 h and were avidly taken up by human monocyte–derived and murine bone marrow–derived macrophages (Fig. 3 a). RGDS peptide and mAbs against macrophage $\alpha_v\beta_3$, CD36, and thrombospondin (TSP) significantly inhibited macrophage ingestion of IL-2–deprived apoptotic CD8+ and CD4+ T cells (Fig. 4, A and B; p <0.001). Phosphatidylserine analogues (11, 12) had no effect on the uptake of human apoptotic CD8+ and CD4+ T cells by human monocyte–derived macrophages (Fig. 4, A and B). Apoptotic CD8+ cells obtained from EBV-infected individuals were recognized and ingested by 35 ± 4% of murine bone marrow–derived macrophages (mean ± SD%; n = 5) that also employ vitronectin receptor–dependent mechanisms (7). This was inhibited by 2 mM RGDS peptide (to 6.4 ± 1.5%) but not by 2 mM RGES (32.6 ± 5.4%). Rabbit polyclonal antiserum to murine TSP 1 was also inhibitory (11.8 ± 3.1%) while the preimmune serum used at the same dilution was not (34.7 ± 1.6%).

We found that apoptotic CD8+ T cells that had been ingested by macrophages retained immunological reactivity for the granule component TIA-1 comparable with apoptotic cells tested before interaction with macrophages (Fig. 3 b).

Macrophage Phagocytosis of Apoptotic T Cells In Vivo. The analysis of lymph nodes taken from HIV+ patients with low CD4 numbers showed that >93% of cells in the paracortex were CD3+,CD8+ and <2% CD4+. PI staining of the paracortex of HIV lymph nodes revealed the presence of fragmented nuclei (Fig. 3 c; asterisks). Two-color immunofluorescence indicated that the majority of these apoptotic cells were CD8+ (not shown). Furthermore the CD68+ macrophages in the paracortex of HIV+ lymph nodes contained condensed/degraded nuclear debris identifiable by PI staining (Fig. 3 c, arrows) suggesting that apoptotic T cells were being cleared by activated macrophages in vivo.

Discussion

In this study we demonstrate that CD8+,CD45RO+ T cells undergoing apoptosis are specifically recognized and phagocytozed by macrophages in vitro and show that the ingested cells are intact within these phagocytes as they still contain cytotoxic granule proteins. Human monocyte–derived macrophages, the phagocytes used in this study, employ two surface receptors in the uptake of neutrophils undergoing apoptosis: the $\alpha_v\beta_3$ integrin and CD36 (7). These receptors cooperate in binding macrophage-secreted thrombospondin, which forms a "bridge" between the macrophage and the apoptotic cell (10). We now show that that these macrophages employ an identical mechanism in the recognition of CD8+ and CD4+ T cells undergoing apoptosis.
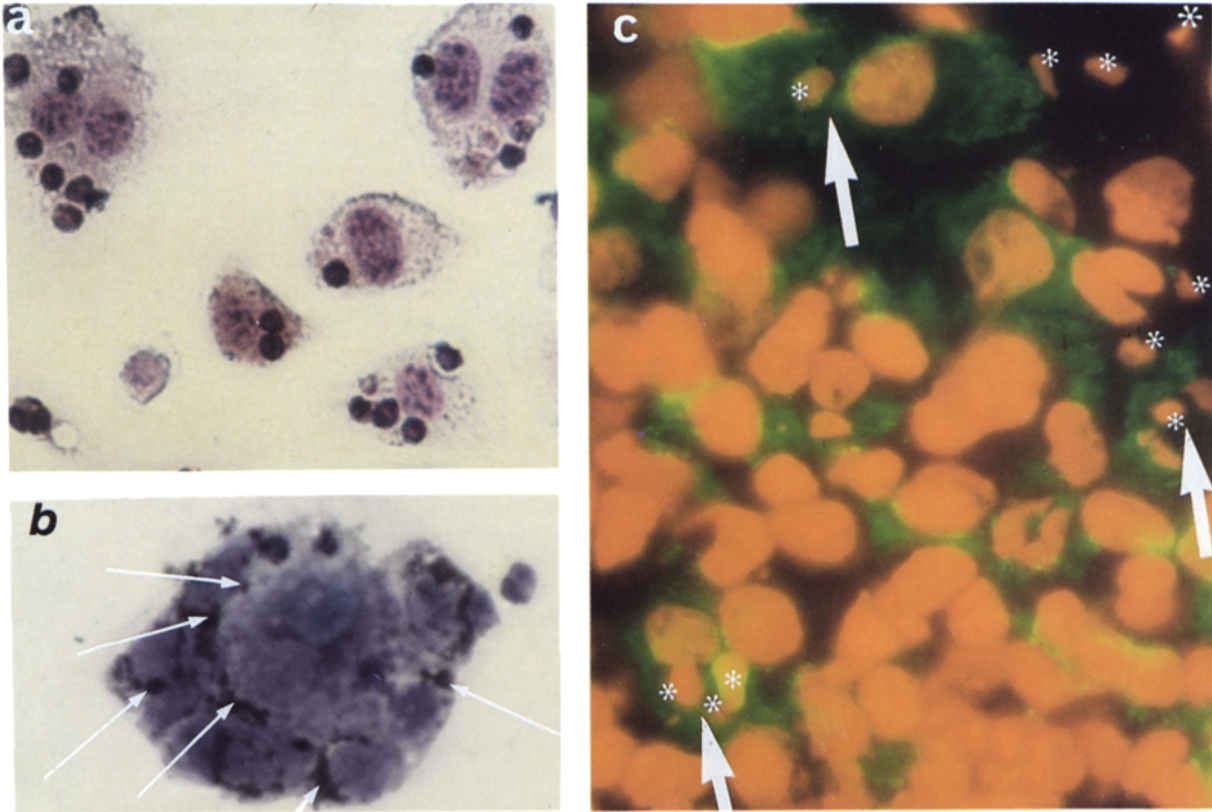
Figure 3. The phagocytosis of apoptotic CD8⁺ T cells by human macrophages. Apoptotic CD8⁺ T cells were incubated with human monocyte–derived macrophages for 30 min followed by washing to remove noningested cells. In *a*, the fixed cells were then stained with hematoxylin & eosin. Ingested apoptotic cells are apparent as pyknotic masses within macrophages (*a*). In *b*, macrophages and apoptotic CD8⁺ T cells were cocultured as above and cytocentrifuge preparations stained for TIA-1 by immunoperoxidase methodology. One macrophage that has ingested numerous apoptotic cells is shown. Macrophages cultured without apoptotic CD8⁺ T cells do not contain TIA-1 (not shown) whereas those with ingested CD8⁺ T cells exhibit granular TIA-1 reactivity (*arrows*). *c* shows the paracortex of a lymph node section from a HIV⁺ patient. In this area, the staining of serial sections indicated that >93% of the cells were CD3⁺,CD8⁺ (not shown). Nuclei were stained with propidium iodide (*orange*) and macrophages were stained with CD68 antibody (*green*). Apoptotic CD8⁺ T cells, indicated by asterisks, are identifiable within macrophages (see *arrows* in *c*).
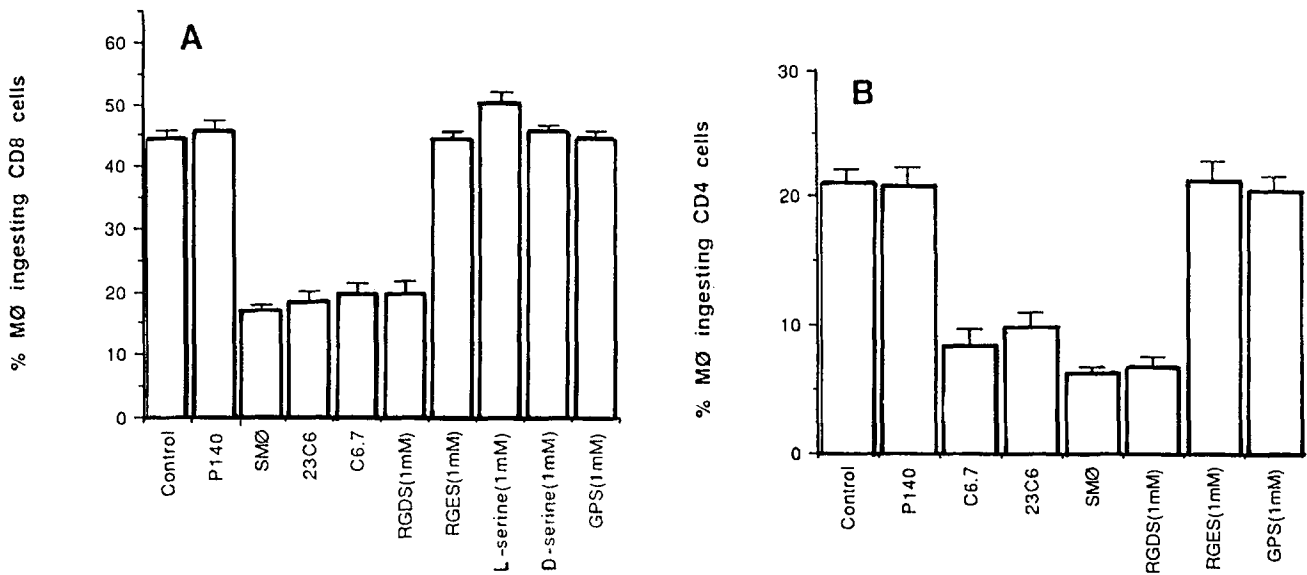

Figure 4. The inhibition of phagocytosis of apoptotic T cells by human monocyte–derived macrophages. Apoptotic CD8⁺ (*A*) or CD4⁺ (*B*) T cells generated in vitro were interacted with human monocyte–derived macrophages. Phagocytosis was inhibited by mAbs to CD36 (Smφ), αᵥβ₃ integrin (23C6), and TSP (C6.7) but not by the irrelevant control antibody P140. Furthermore, RGDS peptide, but not RGES, inhibited the uptake of apoptotic T lymphocytes. Inhibitors of phosphatidylserine-dependent recognition of apoptotic cells (11, 12) such as phosphatidyl-L-serine (depicted as L-serine) and glycerophosphorylserine had no effect; phosphatidyl-D-serine was included as a noninhibitory control. Similar results were obtained with apoptotic CD4⁺ T cells generated in vitro (*B*). The data are shown as mean percentage of macrophages ingesting apoptotic lymphocytes ± SEM (*n* = 10).

A recent report suggests that murine peritoneal macrophages elicited by thioglycollate, which employ the phosphatidylserine receptor in the uptake of senescent cells (11, 12), may be able to recognize leukocyte surface changes that arise as a consequence of 24 h of culture rather than apoptosis (13). In contrast, a close correlation between apoptosis and recognition was found when human monocyte–derived macrophages that employ the $\alpha_v\beta_3$/CD36/TSP receptor system were used in phagocytic assays (7). Furthermore, in the current study, human monocyte–derived macrophages did not recognize nonapoptotic T lymphocytes from healthy donors despite prior culture of these cells in the absence of exogenous stimuli for 24 h.

Patients with HIV infection have considerably increased proportions of CD3^{+},CD8^{+},CD45RO^{+} T cells in the paracortex of lymph nodes that express low levels of Bcl-2 and phenotypically resemble the CD8^{+} subset from EBV patients (4, 14). Extensive nuclear condensation and fragmentation of cell nuclei was observed in the paracortex of HIV^{+} lymph nodes and many of these apoptotic cells were found within macrophages. Although these investigations do not address the viral specificity of the dying cells and if they are directed to HIV itself or other opportunistic infections, they do suggest that the phagocytic clearance of T cells in vitro have a counterpart in vivo. A proportion of the apoptotic cells observed might also have been CD4^{+} because HIV infection may trigger apoptosis in both resting and activated CD4^{+} T cells (15). Our data obtained from experiments in vitro suggest however, that both apoptotic CD4^{+} and CD8^{+} T cells would be recognized and phagocytozed by macrophages in an analogous manner.

In conclusion, we have described a general mechanism for the clearance of CD8^{+} effector T cells and activated CD4^{+} T cells during the resolution of viral disease that is likely to play a key role in the safe reestablishment of T cell homeostasis in vivo.

Address correspondence to Dr. A. N. Akbar, Department of Clinical Immunology, Royal Free Hospital School of Medicine, Pond Street, London NW3 2QG, UK.

References

1. Doherty, P.C. 1993. Immune exhaustion: driving virus-specific CD8^{+} T cells to death. Trends Microbiol. 1:207.
2. Miyawaki, T., Y. Kasahara, H. Kanegane, K. Ohta, T. Yokoi, and A. Yachie. 1991. Expression of CD45R0 (UCHL1) by CD4^{+} and CD8^{+} T cells as a sign of in vivo activation in infectious mononucleosis. Clin. Exp. Immunol. 83:447.
3. Rickinson, A.B., D.J. Moss, L.E. Wallace, M. Rowe, I.S. Misko, and M.A. Epstein. 1981. Long-term T-cell-mediated immunity to Epstein-Barr virus. Cancer Res. 41:4216.
4. Akbar, A.N., N. Borthwick, M. Salmon, W. Gombert, M. Bofill, N. Shamsadeen, S. Pett, J.E. Grundy, and G. Janossy. 1993. The significance of low bcl-2 expression by CD45RO T cells in normal individuals and patients with acute viral infection. The role of apoptosis in T cell memory. J. Exp. Med. 178:427.
5. Salmon, M., D. Pilling, N. Borthwick, N. Viner, G. Janossy, P.A. Bacon, and A.N. Akbar. 1994. The progressive differentiation of primed T cells is associated with an increasing susceptibility to apoptosis. Eur. J. Immunol. 24:892.
6. Podack, E.R., J.D.E. Young, and Z.A. Cohn. 1985. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T cell granules. Proc. Natl. Acad. Sci. USA. 82:8629.
7. Savill, J., V. Fadok, P. Henson, and C. Haslett. 1993. Phagocyte recognition of cells undergoing apoptosis. Immunol. Today. 14:131.
8. Savill, J., I. Dransfield, N. Hogg, and C. Haslett. 1990. Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. Nature (Lond.). 343:170.
9. Geisberg, M., J.A. Trapani, and B. Dupont. 1990. Monoclonal antibodies detecting discrete epitopes of human perforin. Tissue Antigens. 35:229.
10. Savill, J., N. Hogg, Y. Ren, and C. Haslett. 1992. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. J. Clin. Invest. 90:1513.
11. Fadok, V.A., D.R. Voelker, P.A. Campbell, J.J. Cohen, D.L. Bratton, and P.M. Henson. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. 148:2207.
12. Fadok, V.A., J.S. Savill, C. Haslett, D.L. Bratton, D.E. Doherty, and P.A. Campbell. 1992. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J. Immunol. 149:4029.
13. Lagasse, E., and I.L. Weissman. 1994. Bcl-2 inhibit apoptosis of neutrophils but not their engulfment by macrophages. J. Exp. Med. 179:1047.
14. Bofill, M., W.M. Gombert, N.J. Borthwick, A.N. Akbar, J. McLaughlin, C. Lee, M.A. Johnson, A. Pinching, and G. Janossy. 1994. Histological studies of CD3^{+},CD8^{+},bcl-2^{low} lymphocytes in the lymphoid tissue of HIV-1^{+} patients. Am. J. Pathol. In press.
15. Groux, H., G. Torpier, D. Monte, Y. Mouton, A. Capron, and J.C. Ameisen. 1992. Activation-induced death by apoptosis in CD4^{+} T cells from human immunodeficiency virus-infected asymptomatic individuals. J. Exp. Med. 175:331.