Brief Definitive Report

bcl-2 Inhibits Apoptosis of Neutrophils but Not Their Engulfment by Macrophages

By Eric Lagasse and Irving L. Weissman

From the Department of Pathology and Developmental Biology, Stanford University School of Medicine, Stanford, California 94305

Summary

Neutrophils, the most common inflammatory leukocytes, have the most limited life span of all blood cells. After they undergo apoptosis, they are recognized and engulfed by macrophages. bcl-2, a proto-oncogene rearranged and deregulated in B cell lymphomas bearing the t(14;18) translocation, is known to inhibit programmed death. bcl-2 expression is localized in early myeloid cells of the bone marrow but is absent in mature neutrophils. Transgenic mice that expressed bcl-2 in mature neutrophils showed that bcl-2 blocked neutrophil apoptosis. Despite this, homeostasis of neutrophil population is essentially unaffected. In fact, macrophage uptake of neutrophils expressing bcl-2 still occurred. This transgenic model indicates that the mechanism that triggers phagocytosis of aging neutrophils operates independently of the process of apoptosis regulated by bcl-2.

The hematolymphoid system maintains an equilibrium between cell proliferation and cell death. In bone marrow, the majority of proliferating cells are immature neutrophils. They mature into blood neutrophils that are programmed to die rapidly and are cleared by tissue macrophages (1, 2). This rapid turnover is thought to play a critical role in the generation and cessation of an effective inflammatory reaction. Indeed, during acute inflammation massive waves of neutrophils first infiltrate the injured tissue, where they have a major role in the initiation and amplification of inflammation (3, 4). The subsequent clearance of neutrophils from the inflamed site is imperative for resolution to occur. The observation of macrophages bearing engulfed intact neutrophils in inflamed tissues has led to the conclusion that phagocytosis of neutrophils represents the major route for neutrophil disposal (5, 6). In addition, a close correlation has been found between the degree of apoptosis in aging neutrophil populations and the degree of their phagocytosis by macrophages, suggesting that apoptosis of the neutrophil signals the macrophage to engulf it (7–11). bcl-2, an oncogene rearranged and deregulated in B cell lymphomas bearing the t(14;18) translocation (12–14), can block the molecular processes of physiological cell death (15). In transgenic mice, overexpression of bcl-2 in B and T lymphoid cells inhibits their apoptosis and enhances their survival capacity (16). Thus, expression of this gene is a good tool to investigate the role of apoptosis and phagocytosis in relation to homeostasis of neutrophil populations. Since endogenous expression of bcl-2 is restricted to early myeloid cells of the bone marrow but is absent in polymorphonuclear neutrophils of the blood (17, 18), we created transgenic mice expressing bcl-2 in mature neutrophils.

Materials and Methods

All mice were bred and maintained at Stanford University Medical School and their care was in accordance to Stanford guidelines. Transgenic strains were generated from (C3H × C57Bl/6)F_1 hybrid mice and were bred for one or two generations with one of the inbred strains BA or C57Bl/6. For most experiments, transgenic mice and control littermates were 6–10 wk old.

Tissue was isolated from transgenic and littermate controls and treated as frozen sections. Serial sections were fixed in acetone at −20°C for 5 min. Single-cell suspensions collected from peritoneal fluid, blood, or bone marrow were cytospun and fixed as described above before immunohistochemistry.

Mouse neutrophils were isolated from the peritoneal cavity of bcl-2 transgenic and control mice by induction of an inflammation with 2 ml of a 3% thioglycollate medium (Difco Laboratories, Inc., Detroit, MI). Peritoneal exudate cells were harvested 4 h later by peritoneal lavage with PBS solution containing 5 mM EDTA. Peritoneal cells were counted and incubated in a 250-ml flask. Nonadherent cells transferred after 2 h to separate neutrophils from adherent peritoneal macrophages. The neutrophils were cultured in RPMI 1640 containing 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Mouse peritoneal macrophages were isolated after induction of an inflammation in C57Bl/6 as described previously. Peritoneal exudate cells were harvested 4 d later by peritoneal lavage with PBS solution containing 5 mM EDTA. Peritoneal cells were plated in 24-well plates 10^6 at cells/well, and nonadherent cells washed after 2 h. The macrophage monolayers were cultured overnight in RPMI 1640 containing 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin.

For fluorescence-activated flow cytometry analysis, single-cell suspensions were stained with appropriate antibodies, and analyzed by FACScan® (Becton Dickinson & Co., Mountain View, CA).

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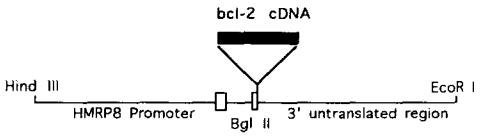

Figure 1. HMRP8/bcl-2 construction. Human bcl-2 cDNA (*filled box*) was inserted in the Bgl II site between the second untranslated exon (*open boxes*) and the 3' untranslated region of the human MRP8 gene (20). The 4.5-kb HindIII-EcoRI fragment was used for microinjection.

Cells were resuspended in staining medium (PBS supplemented with 2% of FCS) containing propidium iodide (PI; 5 μg/ml). More than 10,000 cells were analyzed per sample. Dead cells stained with PI were gated out at the time of analysis.

The phagocytic assay was prepared as described previously (19) Briefly, neutrophils and macrophages were isolated during thioglycollate-induced inflammation. Freshly isolated neutrophils or neutrophils aged in culture (16 mice total, 5×10^6 neutrophils/mouse) were resuspended in 1 ml of medium and then added to each washed well of peritoneal macrophages (10^6 macrophages/well for a 24-well plate cultured for 2 d. The macrophage monolayers were a mixture from two to three different C57Bl/6 donors.) After a 30-min incubation at 37°C in a 5% CO_2 atmosphere, the wells were vigorously washed with cold (4°C) PBS, fixed with 2% formaldehyde in PBS, and then stained for myeloperoxidase (6). Macrophages were scored as positive if they contained one or more myeloperoxidase-positive neutrophils.

Results and Discussion

The transgene was constructed by inserting human bcl-2 cDNA under the control of the human MRP8 regulatory regions (20) (Fig. 1). Four lines of transgenic mice were established. Only one line was used extensively for this study and the others, which have the same phenotype, were reserved for verification of key findings. Tissue-specific expression of the transgenic bcl-2 was examined by immunohistochemistry. Human bcl-2 expression was present in bone marrow, the spleen (Fig. 2), and blood (not shown). The MRP8 gene is known to be strongly expressed in stages of early myeloid cells to blood neutrophils and monocytes, but not in tissue macrophages (21). Analysis of bone marrow and blood cells

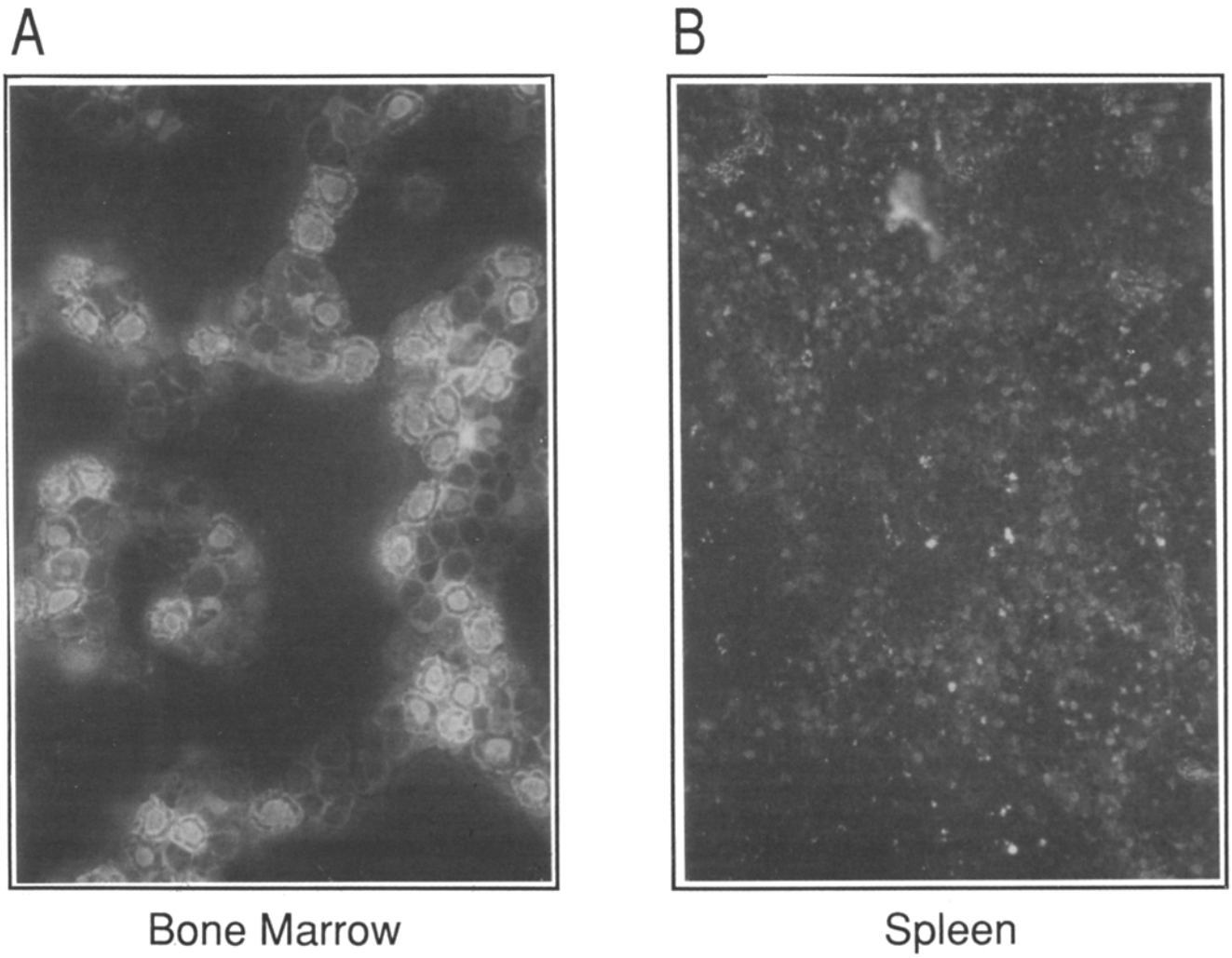

Figure 2. Human bcl-2 expression in transgenic mice. (*A*) Bone marrow cells and (*B*) frozen sections of spleen were stained with anti-human bcl-2 mAb (Dako Corp., Carpinteria, CA). Control littermates demonstrated no reactivity (data not shown).

confirmed that human bcl-2 expression was limited to myeloid and neutrophil cells. In the spleen, only the red pulp and marginal zone were positive, consistent with the expression pattern described for MRP8 and other myeloid markers. Peritoneal and bone marrow macrophages were always negative for transgenic bcl-2.

To assess the effects of bcl-2 expression on apoptosis, neutrophils were isolated from the peritoneal cavity during thioglycollate-induced inflammation, and placed in culture. In culture, neutrophils normally undergo spontaneous programmed cell death characterized by morphological changes including nuclear pyknosis, chromatin condensation, and cytoplasmic vacuolation (22). However, after 24 h of culture, transgenic neutrophils expressing bcl-2 demonstrated markedly increased viability compared with control neutrophils (Fig. 3 *A*). After 3 d in culture, bcl-2-expressing neutrophils remained viable whereas normal neutrophils did not survive. Fluorescence-activated flow cytometry analysis at day 3 confirmed the persistence of the bcl-2 neutrophil population in culture (Fig. 3 *B*). Furthermore, transgenic neutrophils still stained for bcl-2 protein after several days in culture and did not display any of the morphological changes of apoptosis. Neutrophils undergoing apoptosis usually exhibit chromatin fragmentation in a characteristic internucleosomal pattern representing endogenous endonuclease activation (7). As shown by agarose gel electrophoresis of DNA extracted (Fig. 3 *C*), fragmentation was inhibited in neutrophils expressing bcl-2, consistent with their enhanced survival capacity. In summary, overexpression of human bcl-2 blocked programmed cell death of neutrophils in vitro.

Since the survival of the transgenic neutrophils was enhanced dramatically, it seemed likely that neutrophils homeostasis would also be affected. However, flow cytometry analysis revealed that the proportion of neutrophils in the blood did not differ significantly between transgenic and control mice (Table 1). The same was true for the total number of neutrophils (not shown) and the proportion of myeloid cells in the bone marrow and the spleen (Table 1). Therefore, constitutive expression of bcl-2 during myeloid cell maturation did not result in the accumulation of neutrophils. Since the

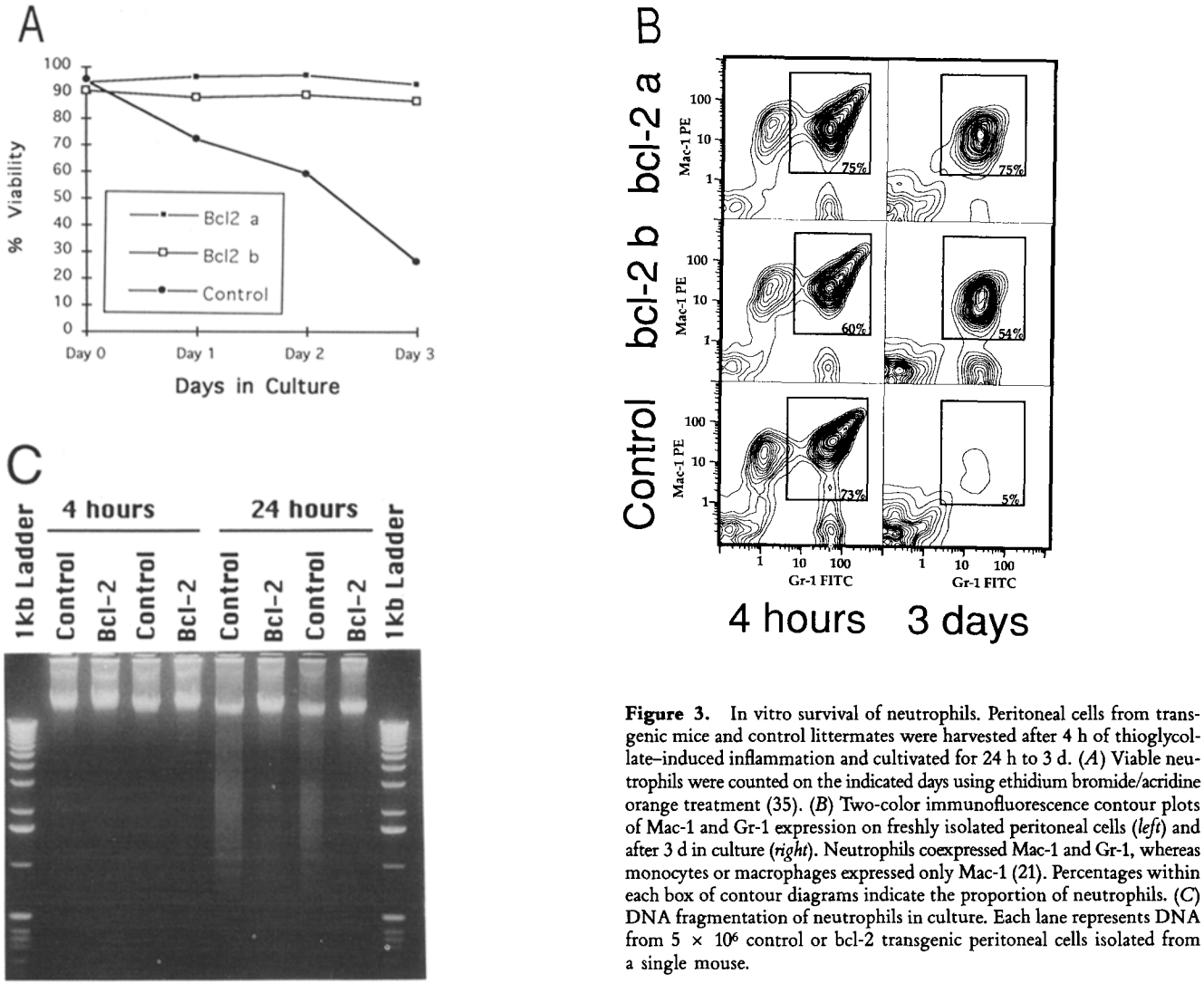

Figure 3. In vitro survival of neutrophils. Peritoneal cells from transgenic mice and control littermates were harvested after 4 h of thioglycollate–induced inflammation and cultivated for 24 h to 3 d. (*A*) Viable neutrophils were counted on the indicated days using ethidium bromide/acridine orange treatment (35). (*B*) Two-color immunofluorescence contour plots of Mac-1 and Gr-1 expression on freshly isolated peritoneal cells (*left*) and after 3 d in culture (*right*). Neutrophils coexpressed Mac-1 and Gr-1, whereas monocytes or macrophages expressed only Mac-1 (21). Percentages within each box of contour diagrams indicate the proportion of neutrophils. (*C*) DNA fragmentation of neutrophils in culture. Each lane represents DNA from 5 × 10^6 control or bcl-2 transgenic peritoneal cells isolated from a single mouse.

Table 1. *Neutrophil Content in the Bone Marrow, Blood and Spleen of Control and Transgenic Mice*

Mice	Bone marrow	Blood	Spleen
Nontransgenic	30.4 ± 4.0	5.8 ± 2.8	1.8 ± 0.4
Transgenic	30.3 ± 8.3	12.6 ± 3.8	2.2 ± 0.4

Neutrophils were counted by flow cytometric analysis of cells bearing Mac-1 and Gr-1 using two-color immunofluorescence. The results are expressed as arithmetric means (three mice) ± SD.

steady state number of neutrophils appeared unaltered in spite of their increased viability in vitro, we decided to examine their fate in vivo. An acute inflammation was induced by injecting thioglycollate broth into the peritoneal cavity of 6-wk-old transgenic mice and control littermates (21, 23). Cells were isolated from the peritoneal cavity at different times and analyzed by flow cytometry (Fig. 4). Several hours after the induction of inflammation, neutrophils accumulated at the inflamed site and became the predominant cell type. After 12–24 h, the number of neutrophils gradually decreased and were replaced by macrophages in both transgenic and control mice. The resorbtion of neutrophils in the transgenic animal was consistently slower than for control mice and the difference was more marked in older animals (not shown). After 2 d, there were no neutrophils left at the site of the inflammation.

To investigate the relationship between neutrophil apoptosis and phagocytosis, we used an in vitro neutrophil–macrophage interaction assay (6, 19). It has been previously reported that neutrophils aged for 24 h in culture spontaneously undergo apoptosis, and are concurrently recognized and engulfed by macrophages (7). Neutrophils from control and transgenic mice were cultivated for 24 h and offered to thioglycollate-elicited macrophages. Phagocytosis of aged control and aged bcl-2 expressing neutrophils was identical (Fig. 5). However, freshly isolated neutrophils were not phagocytosed

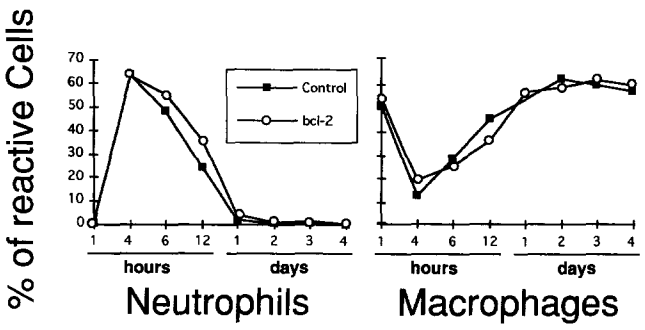

Figure 4. Neutrophil and macrophage populations during thioglycollate-induced peritoneal inflammation. Inflammation was elicited in the peritoneal cavities of control or bcl-2 transgenic mice using 3% thioglycollate broth (21). Exudate cells were harvested on the days indicated. The proportion of neutrophils and macrophages was calculated by flow cytometric analysis of cells bearing Mac-1 and Gr-1 (neutrophils) or only Mac-1 (macrophages) using two-color immunofluorescence.

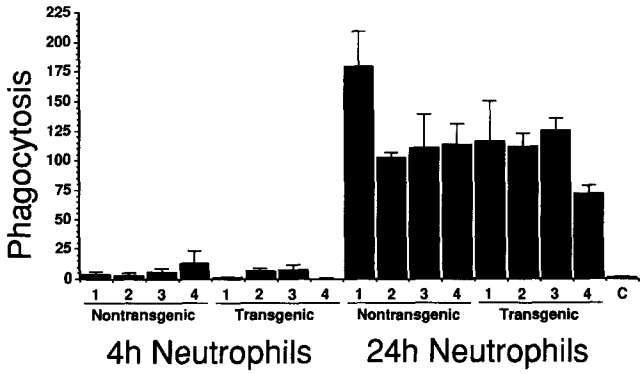

Figure 5. Phagocytosis of neutrophils by macrophages. Phagocytosis was evaluated by counting the number of macrophages ingesting neutrophils using inverted light microscopy. Each count corresponded to the mean ± SD of five different randomly selected microscope fields per well. In the control (lane *C*) no neutrophils were added.

by the macrophages. Thus, although thioglycollate-induced macrophages could not distinguish apoptotic from nonapoptotic neutrophils, they could distinguish aging neutrophils from freshly isolated neutrophils. This result suggests that, independently of apoptosis, neutrophils exhibit cell surface changes that allow them to be recognized and engulfed by macrophages.

These experiments show that bcl-2 can inhibit programmed cell death of neutrophils, but does not prevent them from being engulfed by macrophages. Neutrophil recognition and ingestion by macrophages appears to be a bcl-2–independent pathway and represents the primary mechanism capable of promoting resolution of inflammation. Although neutrophil apoptosis is not a prerequisite for engulfment by professional phagocytes, it may still represent an important event to isolate the cell from its external milieu and control its histotoxic products (24). Earlier studies of bcl-2 forced expression during T cell development reported inhibition of multiple forms of apoptosis but not negative selection of thymocytes (16). This is at least one other cell-disposal mechanism upon which bcl-2 has no effect. Negative selection has been reported to be regulated by thymic antigen presenting cells and cells from the macrophage lineage (25–28). Thus, both neutrophil clearance and negative selection of thymocytes might be regulated by a similar mechanism of recognition and phagocytosis. The genetic pathway by which cell death occurs has been extensively characterized in *Caenorhabditis elegans* (29, 30). In this organism, many extra cells survive when programmed cell death is blocked by overexpression of ced-9 (31) or bcl-2 (32). Since *C. elegans* does not possess a specialized phagocyte resembling the macrophage, the mechanism of recognition may differ somewhat from mammalian system. Nevertheless, seven genes were identified for the engulfment of cell corpses during programmed cell death in *C. elegans* (33) and recently, several classes of receptors have been implicated in the phagocytosis of the senescent neutrophil (34). Further characterization and clarification of the role of these molecules will give a better understanding of immune system homeostasis.

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Address correspondence to Eric Lagasse, Department of Pathology and Developmental Biology. Beckman Center B261, Stanford University School of Medicine, Stanford, CA 94305.

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