Methods. WT mice and mice lacking MMP-2 (gift from S. Itohara, Institute of Physical and Chemical Research, Brain Science Institute, Wako, Japan; 100% C57Bl6) and MMP-12 (gift from S. Shapiro, Harvard Medical School and Massachusetts General Hospital, Boston, MA; 50% C57Bl6 × 50% Swiss) were used. For all experiments, age-, sex- and strain-matched littermate mice were used. Mice were maintained in high-efficiency particulate air (HEPA)–filtered individually ventilated cage (IVC) units. All experiments were performed according to the guidelines for care and use of laboratory animals approved by the institutional ethical animal care committee. Mice were injected with an intravenous bolus of 5-FU (200 mg/kg; Fluroblastin; Pfizer SA/NV, Brussels, Belgium). Peripheral blood was repetitively sampled by retro-orbital puncture under light anesthesia, and full blood counts (ethylenediaminetetraacetic acid [EDTA]–buffered) were determined on a hemocytometer (Cell-Dyn 1300; Abbott, Abbott Park, IL). Doxycycline was administered via drinking water protected from light (30 mg/kg), as described.2

Mice were killed by cervical dislocation. The femurs were removed, fixed in 2% paraformaldehyde in phosphate-buffered saline for 24 hours, and decalcified in 0.5 M EDTA solution for 8 days. After dehydration and paraffin embedding, 10-µm longitudinal sections were prepared on Superfrost Plus slides (Thermo Scientific, Braunschweig, Germany). Immunohistochemistry was performed using antibodies against MMP-2 (Santa Cruz Biotechnology, Santa Cruz, CA) and MMP-12 (R&D Systems, Minneapolis, MN). Specificity for MMP staining was performed using deficient mice (not shown). Corresponding secondary antibodies labeled with horseradish peroxidase or biotin for signal amplification via tyramide signal amplification (TSA; PerkinElmer, Waltham, MA) or via Vectastain ABC kit (Vector Laboratories, Burlingame, CA) were used. For light microscopy, sections were developed with 3,3’-diaminobenzidine (DAB; Sigma-Aldrich, Bornem, Belgium) as a chromogen and counterstained with Harris hematoxylin. Analysis was performed on a Leica Axioplan2 connected to a 3CCD video camera (DNC-930P, Sony, Londerzeel, Belgium), and KS300 software (Zeiss, Zaventem, Belgium).

We used SPSS version 11.0 for statistical calculations. Unless stated otherwise, data (mean ± SEM) were statistically analyzed by an unpaired Student t test. To determine the genotypic differences in WBC counts after 5-FU, an ANOVA for repeated measurements was used, complemented with a t test to identify statistically significant genotypic differences at each individual time point. A P value less than .05 was considered statistically significant.

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References

To the editor:

Granule-mediated death by cytotoxic lymphocytes does not require mitochondrial polarization toward the immunologic synapse in target cells

A recent article in Blood1 reported that mitochondrial polarization to the immunologic synapse (IS) was required for cytotoxic lymphocyte (CL)–induced death of cells that overexpress Bcl-2. This potentially represented a significant advance in understanding CL-induced death because there is currently no way to determine whether a given CL has made a productive killing synapse (that is, whether any granule contents were transferred to the target cell) unless the target cell goes on to die. Polarization of mitochondria in the target cell would provide an early surrogate assay for this process.

We have been following CL-induced death using time-lapse microscopy and find no evidence to support mitochondrial polarization in target cells. To ensure that our experiments had sufficient temporal and spatial resolution, we followed KHYG1-induced death of HeLa cells stained with MitoTracker Red (Molecular Probes, Eugene, OR), and acquired images every 2 minutes (Figure 1A). For analysis, we segmented the cell into four regions, and designated quadrant 1 as the quadrant where the effector cell made contact with its target. The other quadrants were labeled in a clockwise manner. If mitochondria polarized toward the IS, we would expect to observe increased fluorescence in quadrant 1 and a corresponding decrease in fluorescence in quadrant 3. In contrast, we observed a decrease in fluorescence in quadrants 1, 2, and 3 and a corresponding increase in fluorescence in quadrant 4 (Figure 1B). Further, these changes coincided with the target cell rounding and
moving into quadrant 4, rather than polarization of the mitochondria within the cell.

To determine whether the data using KHYG1 cells was generally applicable, we followed HeLa-Bcl-2 cells killed by natural killer (NK) cells isolated from human patients, and MC57-Bcl-2 or MS9II-Bcl-2 cells killed by NK cells isolated from C57BL/6 mice.
directly after CL engagement (Post-hit), directly before the target cell rounded up (Pre-rounding), and directly after the target cell rounded (Round). We clearly detected mitochondrial movement when individual target cells rounded up (Figure 1C), but we consistently found equal distribution of mitochondria in all quadrants before target cell rounding (Figure 1D). Mitochondrial polarization in the target cell is therefore not an early event, generally required for CL-induced killing of Bcl-2—overexpressing cells.

Our conclusions contrast with those of Goping et al. One explanation for this may be that the previous study analyzed suspension cells at only one time point. The mitochondria in these cells could not be followed over time, and it was not possible to determine whether the conjugates analyzed formed a killing synapse. Our study investigated adherent cells where mitochondria could be tracked in real time, and the conjugates analyzed verifiably involved a killing synapse; parameters that are critically important to objectively address this issue. It does remain possible, however, that mitochondrial polarization is required in some, but not all, models of CL-induced death of cells that overexpress Bcl-2.

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Reference

Response

Movement of mitochondria during CTL-mediated killing

The pathways that regulate apoptosis are complex and multilayered. It is well known, for example, that death induced by Fas can in some cells involve mitochondria, while in others does not. In the granzyme system there are cell lines that die primarily through the granzyme B pathway and those that are sensitive to granzyme A. Consequently, we do not find it too surprising that Waterhouse et al have found lines that do not polarize mitochondria.

Clearly there are differences between the target cells used in either study. For example, the kinetics of apoptosis induction differ markedly in the 2 systems. We see mitochondrial membrane potential loss very early in our cells, whereas they state that it is more than an hour before they observe cytochrome c loss, although this is difficult to appreciate from their data as the cc–green fluorescent protein (GFP) appears throughout the cell at time zero. It is also unclear what effector molecules are involved, as in their previous work Waterhouse et al have reported that some of their killer lines use primarily perforin. In addition, one interesting difference between our targets and those used by Waterhouse et al is the high density of mitochondria in the HeLa cells. Clearly the HeLa already have a large number of mitochondria adjacent to the synapse.

Waterhouse et al imply that our work is less reliable because we used single time points and cannot determine whether target cells eventually undergo apoptosis. The advantage of our
Granule-mediated death by cytotoxic lymphocytes does not require mitochondrial polarization toward the immunologic synapse in target cells

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