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By the 1990s, it was well established that many cells die during the normal course of events in developing organisms and that cell death occurs in many different ways. Despite this, however, the corpses of these dead cells are usually not apparent. This is particularly true of the immune system. It was known that every day, mammalian hosts produce many T and B cells and, for that matter, neutrophils, yet the numbers of these cells in the hosts do not change appreciably as adults age. Therefore, cells must somehow die and subsequently be cleared rapidly. Not only that, but by the 1990s, it was known that, as predicted by Lederberg (1), autoreactive T cells and some autoreactive B cells die in the organs that produce them: the thymus and bone marrow (2–4). Despite this, the corpses resulting from this phenomenon could not be found in thymus or bone marrow.

The process of apoptosis (and its characteristic creation of DNA fragments in apoptotic cells) was described before the early 1980s (5–7). However, the detection method involved bulk cells and did not reveal where the dying cells were within tissues or whether the cells had been engulfed by others. The TUNEL method, invented by Gavrieli et al. (8), was therefore a welcome addition to scientists' armamentaria. In their paper on the subject, Gavrieli et al. used the method to show, for example, that the dying cells in the rapidly turning over intestinal epithelium were at the tips of the intestinal villi, where they could presumably be shed into the gut lumen or be engulfed by local cells.

In this *Pillars of Immunology* article, Surh and Sprent (9) used the TUNEL assay to find the missing and presumed dead lymphocytes of the thymus. The thymus was a particularly interesting organ to choose for their studies because it was already known that thymocytes must die there. Thymocytes are selected for survival based on the affinity/avidity of their receptors for MHC/peptide combinations on epithelial cells in the thymus. Cells bearing receptors that do not react well enough with MHC/peptide die of neglect. Cells bearing receptors that react with too high an affinity/avidity activity eliminate themselves through clonal deletion. Only those cells with affinities/avidities for thymus MHC/peptide within the correct (moderate) range survive (10). The question that Surh and Sprent addressed with the TUNEL/thymus experiments was the following: "Where in the thymus does death by neglect versus death by clonal deletion occur?"

Using thymus sections and the TUNEL method, they found that in normal mice, most apoptotic cells were present in the thymus cortex, engulfed by F4/80$^+$ macrophages, and not engulfed by the more prevalent cortical thymic epithelial cells. They tested whether the TUNEL$^+$ cells in the cortex were dying of neglect or clonal deletion in two ways. First, they used mice lacking expression of both MHC-I and MHC-II. The numbers of TUNEL$^+$ cells in the thymus cortex were unaffected by the absence of MHC. In another set of experiments, Surh and Sprent (9) used mice expressing a Vβ5$^+$ TCR transgene and also expressing, or not, an MHC–IE protein. Vβ5$^+$ cells had previously been shown to react with IE proteins engaged by the endogenous mouse mammary tumor virus 9 superantigen (11). Surh and Sprent found that the numbers of apoptotic cells were increased in the thymus medulla of the IE$^+$ mice, and the apoptotic cells were engulfed by F4/80$^-$MAC-3$^+$ macrophages. They concluded that clonal deletion via this "self" Ag occurs in the thymus medulla, whereas death by neglect happens in the thymus cortex. In their discussion, Surh and Sprent deal with this interpretation guardedly, however, because of their finding that in normal mice, most of the apoptotic cells are in the thymus cortex. Thus, some clonal deletion must occur there, assuming that thymocyte clonal deletion happens in normal mice. Moreover, as Surh and Sprent reference in their paper, other methods had shown that autoreactive T cells can be deleted in the thymus cortex (2, 3, 12). Putting these ideas together, the authors concluded that most deletion in the thymus cortex occurs because of neglect, involving thymocytes that either do not express a functional TCR or whose TCR is of too low affinity/avidity for MHC/peptide to allow positive (or negative) selection.

Later experiments showed that a surprisingly high percentage of TCR$^+$ positively selected thymocytes actually are potentially autoreactive (13). Did the TUNEL assay somehow underestimate the numbers of dying thymocytes? missing, selectively perhaps, those that were dying because they were potentially autoreactive? Does the underestimation account for the fact that the authors found almost no dying cells in the thymus medulla? Possibly. Estimates are that, in a mouse thymus, approximately one third of the cells are produced each day. Of these, only 1–2% per day are exported to the periphery (14, 15). Therefore, approximately one third of the cells must die each day. This is certainly not the impression one gets from looking at the images in the Surh and Sprent paper. The difference is probably due to the fact that the images are just snapshots; dead cells may be completely

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destroyed much more quickly than the images can capture them. The images reveal what is happening at a particular moment but not what is going on in a full 24 h period.

Finally, the approaches were different in the 1990s than in 2018. The Surh and Sprent paper is an illustration of the masterful use of a new technology to deal with an important question, but by present day standards, it is surprisingly lacking in quantitation. We are shown pictures of the thymus to illustrate the sites of cell death, but no quantitative data are given, no "counting of dead cells per field" is performed. These are the types of numerical estimations of the results that would certainly be demanded, perhaps unnecessarily, by a reviewer in 2018.

This *Pillars of Immunology* article is dedicated to the memory of Dr. C. Surh, a wonderful colleague, who died in October 2017.

Disclosures

The author has no financial conflicts of interest.

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