Are apoptotic cells in a PBMC population bystanders or do they interfere with antigen-specific T cell assays?

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INTRODUCTION: As soon as PBMC are isolated, cells start dying. The rate of apoptotic cell death is increased when PBMC are shipped, cryopreserved or stored under suboptimal conditions. Cells undergoing apoptosis are known to secrete mediators such as Lactoferrin that actively suppress inflammation while promoting phagocytosis. The increased presence of apoptotic cell numbers in PBMC therefore may modulate T cell functions in antigen-triggered T cell assays such as ELISPOT or ICS. Also, the presence of increased numbers of apoptotic cells in PBMC indicates that the sample has undergone damage that may affect non-apoptotic T cells as well. However, how reliably does the frequency of apoptotic cells in PBMC reflect functional impairment of the T cell response?

METHODS: In one approach anti-CD20 MAb-coupled beads were used to selectively induce apoptosis in the B cell subpopulation of human PBMC followed by ELISPOT testing. Supernatants of purified B cells undergoing apoptosis were added to PBMC and tested in ELISPOT assays. In a second approach, thawed PBMC were stored overnight at 4°C inducing an increased rate of apoptosis compared to PBMC stored in the incubator overnight. In a third approach, PBMC were stored in DMSO to induce apoptosis. CD8 cell reactivity were tested in an IFN-γ ELISPOT assay with CEF peptide pool as the antigen while normalizing for the live cell count. Live, dead and apoptotic cells were counted using CTL's cell counting platform. IFN-γ was measured using ImmunoSpot® Test Kit, and to assure low background, CTL-Test™ Medium was used. The spots were counted using an ImmunoSpot® S6 Core reader.

RESULTS: Overnight treatment with anti-CD20 antibody induced apoptosis in close to 100% of the B cells. Storage of PBMC overnight at 4°C induced apoptosis in 6% of the PBMC. However, the apoptotic process continued during the 24h of ELISPOT testing resulting in an additional 17% apoptosis. Testing of the PBMC in which B cells were selectively induced to undergo apoptosis showed that the CEF peptide specific ELISPOT counts were unaffected. Also, adding supernatants of apoptotic B cells did not significantly affect the ELISPOT counts. These data argue against mediators released by dying cells (in this case B cells) interfering with CD8 T cell functionality in ELISPOT assays. In contrast, testing of the overnight 4°C treated PBMC or PBMC stored in DMSO, however, showed that the diminution of the T cell response was markedly greater than the decrease in viable cell numbers.

CONCLUSIONS: **(1)** Apoptosis of bystander PBMC does not per se impact CD8 cell function. **(2)** Measuring the number of apoptotic cells before plating the PBMC does not necessarily reflect the extent of cell injury: an increased rate of apoptosis continues in damaged cell samples throughout the subsequent T cell assay. **(3)** Measuring the number of apoptotic cells during and after the T cell assay reveals damage to the PBMC, but the functional deficiency of T cells is higher than indicated by the rate of apoptosis. **(4)** T cell functionality is impaired after cellular injuries that are not strong enough to lead to cell death. In addition to measuring live, dead and apoptotic cells in PBMC, additional markers are needed that would identify sublethal damages to the cells that lead to functional deficiency, and therefore help assess whether a PBMC sample is suitable for functional T cell assays.

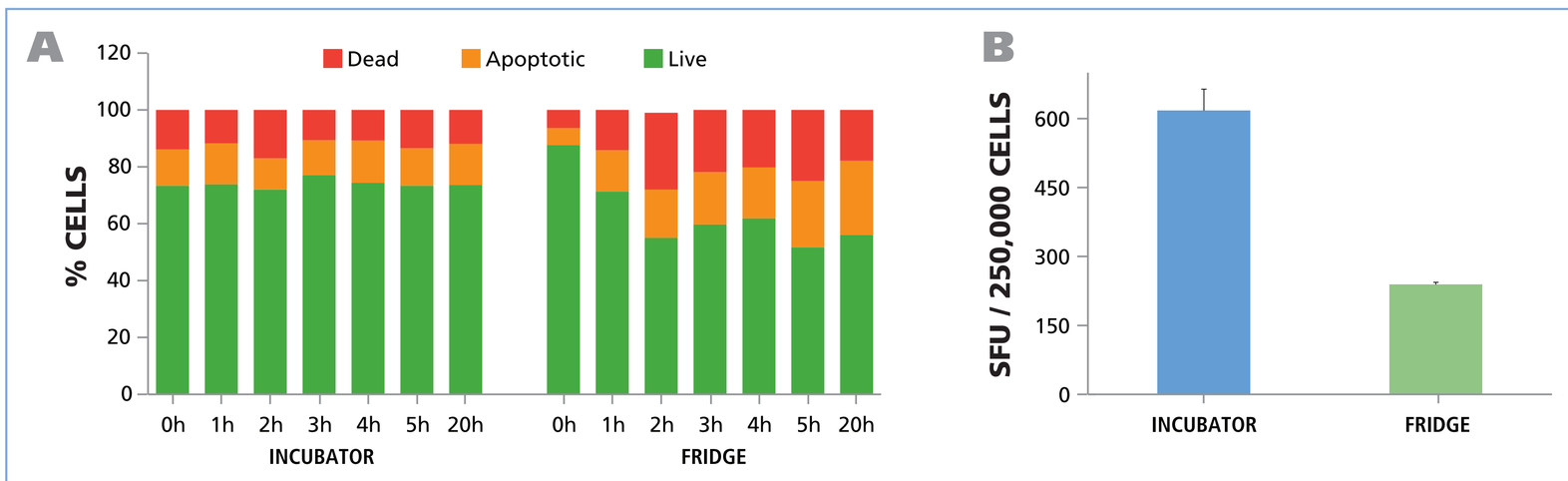


Figure 2: **Apoptosis induction by PBMC storage at 4˚C.** PBMC were stored at 4˚C ("Fridge"), or at 37˚C ("Incubator") overnight. (A) The numbers of live, dead and apoptotic cells were counted starting after the overnight storage at the specified time points. During this assessment, the cells were kept in an incubator. The data show an increased apoptotic rate for the cells previously stored at 4˚C, that initially is not detectable, but becomes evident at later time points. (B) The PBMC were resuspended in fresh medium after the overnight storage in the incubator or the fridge, and tested in an IFN-γ ELISPOT assay with CEF peptides as the antigen. Although viability of cells were similar when recorded at the start of the assay (see A), the T cell function was greatly reduced for the cells stored in the fridge.

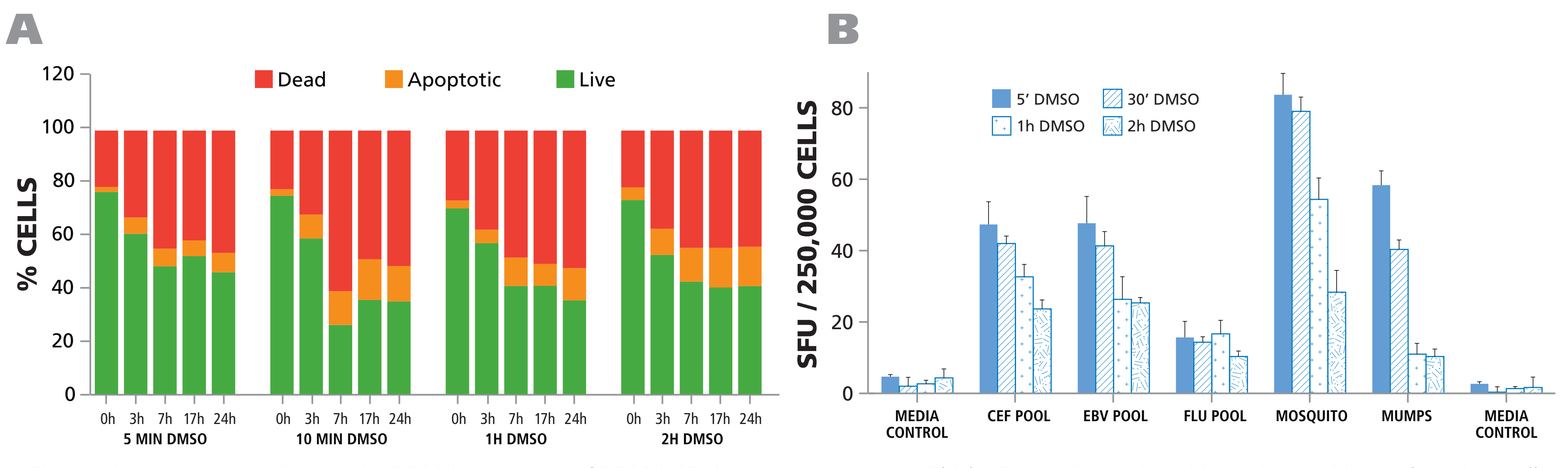
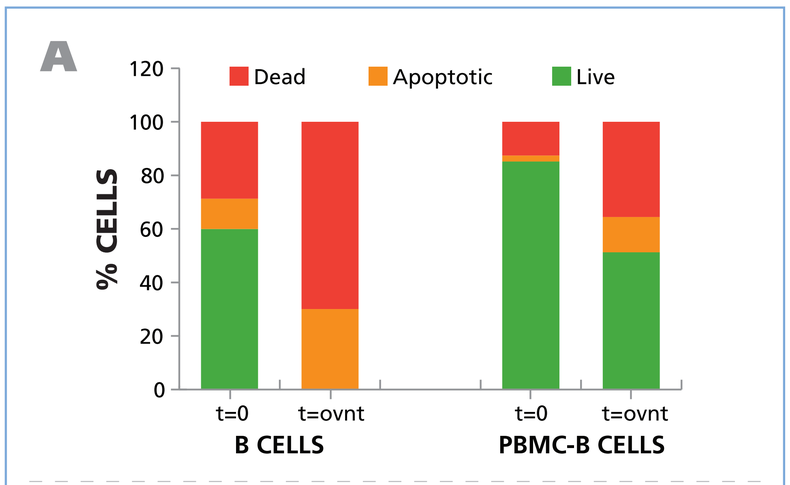


Figure 3: **Apoptosis induction by DMSO exposure of PBMC.** PBMC were incubated at 37˚C for 5min, 10min, 1h and 2h with 10% DMSO after which cells were washed, resuspended in fresh medium, and stored in the incubator while the numbers of live, dead and apoptotic cells was counted at the specified time points (A). After the different DMSO exposure periods, the PBMC were resuspended in fresh medium and tested in an IFN-γ ELISPOT assay with the specified antigens. CEF, EBV and Flu peptide pools activate CD8 cells, Mosquito, and Mumps antigens trigger CD4 cells. In this model too, the viability and apoptosis % determined prior to the start of the assay were similar for all 4 DMSO treatment groups, yet the functionality was largely different.



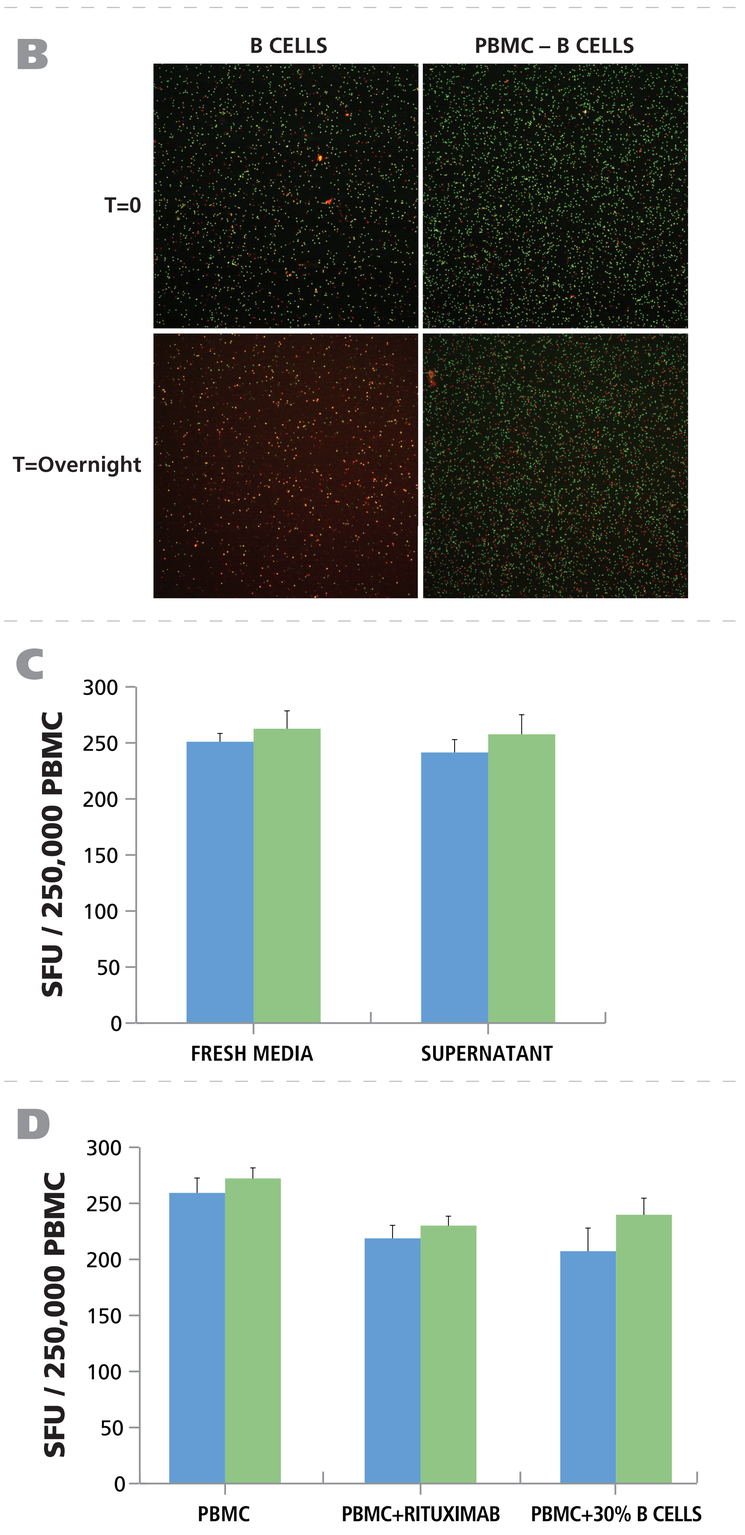


Figure 1: **Apoptosis induction in B cells by overnight culture with anti-CD20 coupled magnetic beads.** B cells were isolated from PBMC using anti-CD20 coupled magnetic beads and were incubated overnight. The numbers of live, dead and apoptotic cells was established right after separation (t=0) and after an overnight culture. (A) The percentage of live, dead and apoptotic cells are shown for the B cells and the remainder of PBMC. (B) The images of the cells with live cells stained green and dead cells stained red. (C) B cells isolated from PBMC were incubated overnight with monoclonal anti-CD20 antibody (Rituximab) and the supernatant was added to an IFN-γ ELISPOT assay in which PBMC were stimulated with the CEF peptide pool. Spot formation in the presence of fresh media, or of the apoptotic supernatant was compared for two PBMC donors, LP11 and LP31, as specified by the colors. (D) The same two donors were tested when Rituximab was directly added to the PBMC during the ELISPOT assay, or when 30% apoptotic B cells were added. Neither apoptotic B cells, nor their supernatant significantly affected CD8 cell function.

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