Tracking Immune Cells After Tissue Damage Via X-Ray Irradiation in *Manduca sexta*, to Understand the Regenerative Ability of Insect Immune Cells

Rachel Bhaskar

Biology Department

San Francisco State University, San Francisco, California

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Immunological processes influence the overall development of every organism. Innate immune responses drive the sole defensive mechanisms in the Lepidopteran insect host, *Manduca sexta* (Figure 1A). Hemocytes are similar in function to human white blood cells. It is known that *M. sexta* hemocytes (Figure 5), especially plasmatocytes and granulocytes (Figure 3) play a role in encapsulation of foreign bodies and phagocytosis of apoptotic bodies, respectively. These immune cells originate at the hematopoietic organ, which lies above proliferating cells on the wing imaginal discs (Figure 1B). These wing discs are progenitors of the adult wings, and begin to grow rapidly in the last larval stage. Selectively damaging the imaginal discs via x-ray irradiation in the larvae delays development, putatively to provide time for the discs to regenerate. It is suggested in *Drosophila melanogaster* and *Bombyx mori*, that hemocytes play a significant role in the repair of damaged discs (Pastor-Pareja et al. 2008, Tan et al. 2013). Circulating hemocytes adhere to tumors or damaged discs upon detection of basement membrane disruption. Whether a similar role exists in *M. sexta* is still unknown.

To assess changes in hemocyte populations in response to tissue damage, hemolymph (insect blood) was collected from *M. sexta* after inducing tissue damage with 3500 rads of x-rays immediately and 24, 48, and 72h later. We assessed changes in complexity of the hemocyte populations (Figure 2), and then confirmed the cell types using fluorescently labeled hemolymph samples (Figure 3). We noted an increased level of granulocytes in the hemolymph of irradiated larvae (Figure 4). Furthermore, we determined the proliferation rate of new epithelial cells from the imaginal discs after x-ray induced damage by staining the tissue with nuclear S-phase biomarker, BrdU (Bromodeoxyuridine). The preliminary results indicate relatively higher abundance of proliferating cells in the imaginal discs of irradiated animals (Figure 6). Interestingly, this data correlates with increases in circulating hemocytes.

The high level of granulocytes in circulation suggests a surge in apoptotic bodies post irradiation, since granulocytes phagocytose dying cells. Moreover, the maintained abundance of granulocytes in irradiated hemolymph indicates a compensatory circulation of these immune cells as they remain in the defensive state more than 24 hours after surviving tissue damage. In addition, a trans-differentiation of plasmatocytes to granulocytes, which has previously been observed in *Drosophila* (Leitão et al., 2015), may also be occurring in *M. sexta*. Thus, it appears that the localized discs release abundant cells to help regenerate new cells, while signaling granulocytes to obliterate x-ray damaged cells in hemolymph.

The marked difference in the activity of these sub-hemocyte populations shows the specificity of innate immune defenses in insects, and the ability to regenerate cells, possibly via signaling from lymph glands. The integrity of insect defenses is witnessed by the insect’s ability to combat high doses of irradiation, while continuing to metamorphose into viable adults (unpublished data). The results of this study will aid in further use of *M. sexta* as a robust model organism for immunological and molecular signaling research.
Figure 2: Immunolabelled hemocytes were imaged with Nikon TiE-2000S microscope at 40x. (R. Bhaskar, Fuse Lab; pink = plasmatocytes with DAPI-stained nuclei, green = granulocytes, blue = nuclei)

Figure 3: Unstained cells were run through flow cytometry to obtain a dot plot (A) that detects cells based on size and complexity. Regions were analyzed by cell number, and (B) graphs reflect that aggregated cells decrease after irradiation, while complex cells increase significantly 24 and 48h after irradiation. Apoptotic cells increase 0h post irradiation, and decreases 24h post irradiation. (R. Bhaskar, Fuse Lab; *= p<0.01, n =10)
Figure 4: Immunolabelled cells were run through BD FACS Calibur flow cytometer to obtain a (B) dot plot that detects cells based on fluorescence (as described in Nardi et al. 2003). The dot plots are representative data of sample replicates, using which the cell events were gated to present (A) graphical statistics. Regions were analyzed by cell numbers at FL1 (FITC-labeled granulocytes) and FL4 (Alexa Fluor 647- labelled plasmacytes) axes. Granulocytes remain high in irradiated animals, while plasmacytes decrease post irradiation (R. Bhaskar, Fuse Lab; **= p<0.001, *=p<0.01, n =2).

Figure 5: A 3-D holographic image of live hemocytes adhered on a glass slide. (A) Granulocytes and (B) plasmacytes were visualized using the Nano Live Cell Explorer Microscope. (Cell size diameter approx. = 15μm)

Figure 6: Imaginal discs with BrdU stained cells, which label newly proliferating cells at S-phase. 40x images were obtained using Nikon Eclipse 80i microscope (R. Bhaskar, Fuse Lab).