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Space- and Time-Resolved Analysis of NFAT Signaling in Tumor-Specific $CD8^+$ T Lymphocytes through Multiphoton Intravital Microscopy

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Multiphoton intravital microscopy (MP-IVM) has over the past few years drastically changed our view of immunological processes in vivo toward a much more dynamic one by providing the means to directly visualize cell migration and interactions under different immunological conditions in living anesthetized animals. However, observations were so far largely restricted to the characterization of cell motility and cell-cell contacts, while information about the physiological consequences of such contacts (e.g. T cell activation) was typically inferred from correlative ex vivo analysis.

The broad focus of our laboratory is to overcome this limitation, allowing the multiparametric study of cell motility and the triggering of signaling pathways in immune cells in real-time in the living anesthetized animal. To achieve this goal, we exploit fluorescent signaling probes generated through the fusion of a fluorescent protein with regulatory domains of a key signaling protein; as a result, the fluorescent signal changes subcellular localization (e.g. from the cytoplasm to the nucleus) upon triggering of the respective signaling pathway. We use viral vectors to transduce the signaling probes into target cells, which are subsequently transferred into recipient mice and visualized through MP-IVM in physiological contexts. The analysis of imaging data is performed through novel semiautomatic algorithms allowing unbiased analysis of signaling and the automatic correlation with motility parameters.

As an example of the many lines of research currently pursued in the lab, we report the intravital analysis of $CD8^+$ T cell activation, after encounter of the cognate antigen in the lymph node and in the tumor environment. As readout of T cell activation, we use an NFAT-eGFP fusion protein [1], which resides in the cytoplasm of resting cells, but rapidly accumulates in the nucleus upon engagement of the T cell receptor. This probe was expressed in $CD8^+$ T cells specific for the model antigen *Influenza* Hemagglutinin (HA). Preliminary in vitro characterization of the probe was performed by co-culturing NFAT-$eGFP^+$ $CD8^+$ T cells with B cells loaded with graded amounts of the HA peptide. We found that peptide concentrations as low as 100pM induced the complete nuclear localization of the reporter. To quantify the kinetic of T cell activation in vivo, we injected NFAT-$eGFP^+$ HA-specific T cells into mice. Two days later, we injected HA-pulsed B cells, which homed to the lymph nodes and behaved as target for T cells. MP-IVM analysis of lymph nodes was performed according to our established protocol [2]. We found that T cells stably interacted only with HA-pulsed B cells, but not with control B cells, and that full NFAT nuclear translocation occurs within 1.5 minutes after contact formation (Fig. 1). We remark that this is the first measurement of NFAT kinetics in vivo, which reveals faster kinetics than currently estimated based on data from non-physiological systems.

We then analyzed NFAT signaling in tumor-infiltrating T cells. To do so, we implanted dorsal skinfold chambers (DSFC) in mice. DSFCs allow for the longitudinal observation of subcutaneously implanted tumors by MP-IVM over the course of days. We injected CT26 tumor cells expressing the blue fluorescent protein Cerulean and HA ($CT26_{Cer}$-HA), or parental $CT26_{Cer}$ cells as control. Subsequently, we also transferred NFAT-eGFP expressing HA-specific $CD8^+$ T cells, which began to infiltrate tumors in an antigen independent fashion over the first two days. In HA-expressing tumors, nuclear translocation of NFAT-eGFP was readily found in $CD8^+$ T cells interacting with tumor cells, and to a lesser extent after interaction with undefined components of the tumor stroma. NFAT nuclear translocation was accompanied by a strong migratory stop signal. Using mice in which myeloid cells were specifically labeled, we demonstrated that tumor-associated macrophages surrounding the tumor parenchyma were able to cross-present HA to tumor-specific T cells. T cells infiltrating control HA-nonexpressing tumors never displayed NFAT nuclear translocation, and maintained high motility.

In conclusion, we have developed a novel platform for the analysis of signaling in real time, at the single cell level, in the living animal. In the future, this platform will allow for the analysis of a variety of signaling pathways relevant to the function of tumor-specific T cells in a multiparametric fashion. Studying how signaling pathway are coordinated in vivo to promote T cell activation or immune tolerance will shed light on regulation of antitumor immunity, and will help designing improved protocols of tumor immunotherapy.

References

[1] J. Aramburu, *Science*, 285 (1999) 2129.
[2] T.R. Mempel, *Immunity*, 25 (2006) 129.
[3] This research is supported by NIH funding (RO1 CA150975-01 to TRM)

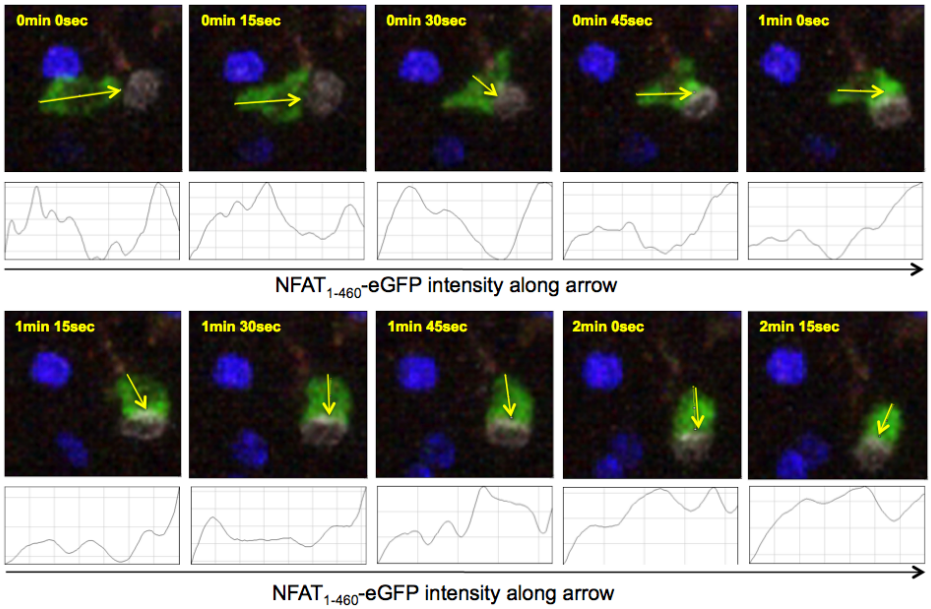

FIG. 1. MP-IVM recording of NFAT-eGFP nuclear translocation in T cells. A HA-specific $CD8^+$ T cell transduced with NFAT-eGFP (green) interacts with a HA-loaded B cell (white) while ignoring a control B cell (blue). After interaction with the HA-loaded target, NFAT translocates to the nucleus within 1.5 minutes. Histograms represent the green fluorescence intensity along yellow arrows.

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