Video Article

Quantitative Imaging of Lineage-specific Toll-like Receptor-mediated Signaling in Monocytes and Dendritic Cells from Small Samples of Human Blood

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Abstract

Individual variations in immune status determine responses to infection and contribute to disease severity and outcome. Aging is associated with an increased susceptibility to viral and bacterial infections and decreased responsiveness to vaccines with a well-documented decline in humoral as well as cell-mediated immune responses[1,2]. We have recently assessed the effects of aging on Toll-like receptors (TLRs), key components of the innate immune system that detect microbial infection and trigger antimicrobial host defense responses[3]. In a large cohort of healthy human donors, we showed that peripheral blood monocytes from the elderly have decreased expression and function of certain TLRs[4] and similar reduced TLR levels and signaling responses in dendritic cells (DCs), antigen-presenting cells that are pivotal in the linkage between innate and adaptive immunity[5]. We have shown dysregulation of TLR3 in macrophages and lower production of IFN by DCs from elderly donors in response to infection with West Nile virus[6,7].

Paramount to our understanding of immunosenescence and to therapeutic intervention is a detailed understanding of specific cell types responding and the mechanism(s) of signal transduction. Traditional studies of immune responses through imaging of primary cells and surveying cell markers by FACS or immunoblot have advanced our understanding significantly, however, these studies are generally limited technically by the small sample volume available from patients and the inability to conduct complex laboratory techniques on multiple human samples. ImageStream combines quantitative flow cytometry with simultaneous high-resolution digital imaging and thus facilitates investigation in multiple cell populations contemporaneously for an efficient capture of patient susceptibility. Here we demonstrate the use of ImageStream in DCs to assess TLR7/8 activation-mediated increases in phosphorylation and nuclear translocation of a key transcription factor, NF-κB, which initiates transcription of numerous genes that are critical for immune responses[8]. Using this technology, we have also recently demonstrated a previously unrecognized alteration of TLR5 signaling and the NF-κB pathway in monocytes from older donors that may contribute to altered immune responsiveness in aging[9].

Video Link

The video component of this article can be found at http://www.jove.com/video/3741/

Protocol

1. Isolation and Stimulation of Immune Cells

1. Obtain 10-50 ml heparinized blood from healthy volunteers after written informed consent under the guidelines of the local Institutional Review Board. For studies of aging or disease pathogenesis, exclusion and inclusion criteria for each volunteer or patient must be explicitly determined.
2. Isolate human peripheral blood mononuclear cells (PBMCs) using CPT tubes or Ficoll-Paque Plus according to the manufacturer's instructions (GE Healthcare, NJ) and conduct experiments on the day of isolation[7] (Reagents, **Table 1**).
3. Incubate PBMCs (3 x 10^6/tube) in 1.5 ml tubes in 0.6 ml medium alone or stimulated with TLR ligands or other activating agents, e.g. the TLR7/8 ligand R848 (0.6 ml medium plus 0.6 µl of 10 mM TLR7/8 ligand R848, final concentration 10 µM) at 37 °C for 10-60 min (InvivoGen, CA)[9].

2. Labeling of Cells

1. Label cell lineage and other surface markers on live cell suspensions using fluorescently-conjugated antibodies. Label 3 x 10^6 PBMCs for 20 min at 4 °C in 100 µl of PBS/2% FBS in a 1.5 ml Eppendorf tube with a cocktail of surface antibodies specific for monocyte or DC lineages (**Table 2**). Optimal antibody dilutions can be determined in preliminary experiments and samples should be processed in batches to minimize

lot-to-lot variation. For vendor antibody stock of 0.1 mg/ml use 5 µl of antibody (1:20 dilution). Use a separate tube of cells labeled with only one single color conjugate to set up the fluorescent channels on the instrument.

2. After surface labeling, fix cells in 4% PFA/PBS for 10 min at RT. For storage until time of analysis, centrifuge cells at 500 x g and resuspend cells in freezing buffer (90% FBS containing 10% DMSO) at -80 °C until the day of assay.
3. For assessment, process batches of untreated and stimulated cells from a group of donors together to minimize variability. On the day of analysis, thaw cells quickly in 37 °C water bath, centrifuge at 500x g to remove freezing buffer.
4. To permeabilize cells for detection of intracellular cytokines or other markers, resuspend cell suspensions in 100 µl BD Perm/Wash buffer (BD Biosciences, NJ). Label for intracellular signaling components with e.g., 1:20 dilution of rabbit anti-NF-κB (p65) antibody (final concentration 10 µg/ml, SantaCruz Biotechnology, CA) for 20 min at RT, centrifuge at 500 x g, aspirate supernatant and resuspend cells in 100 µl BD Perm/Wash buffer containing 1:250 dilution of goat anti-rabbit IgG-Alexa647 (Invitrogen, CA) and incubate for 20 min at RT.
5. Immediately prior to imaging, counterstain nuclei with DAPI (0.2 µg/ml, Invitrogen, CA) or propidium iodide (PI; 20 ng/ml, Invitrogen, CA).

3. ImageStream Analysis

1. Conduct imaging by ImageStream on batched samples to reduce variability between human samples. Instrument settings for power of the lasers were as follows: 200 mW for 488 nm, 10 mW for 658 nm and 250 mW for 405 nm.
2. To analyze data files (**Fig. 1**), first, gate on events with normal PI intensity and high PI aspect ratio (width:height ratio) to distinguish single cells (R1) from debris (low PI intensity) or multi-cellular events (high PI intensity and low aspect ratio). Monocytes are within a gate for CD14 positive cells. mDCs are within a gate for cells that are high for CD11c and low for CD4 (R2) and also low for Lin-1 markers (R3).
3. Use IDEAS software (Amnis, WA) to provide an effective quantitative measure of the degree of activation of each cell. Determine similarity (or co-localization) of the cytoplasmic transcription factor NF-κB with nuclear dye PI to indicate translocation into the nucleus (R4). A high correlation of NF-κB/PI localization is reflected in a high similarity score and indicates the degree of activation[10].
4. Compare the similarity ratios between different treatment groups or patient populations to indicate relative functional efficiency of the cells. Quantify data between samples through statistical comparison of absolute values or fold differences. Use IDEAS software to display images of cells from key segments of population histograms (**Fig. 2**).

4. Representative Results

We have quantified signaling pathways in response to a model viral ligand by stimulating PBMCs with the TLR7/8 ligand, R848 (**Fig. 1**). We collected both cellular localization images and population statistics in our sample groups (**Fig. 2**). ImageStream allows us to gate cell subsets directly from PBMCs and image cell responses from gated, but unsorted cell populations. Here we quantified the effect of TLR signaling on nuclear localization of NF-κB (p65) in Lin1-, CD4 dim, CD11c+ myeloid DCs (mDCs). At baseline, we observed a high percentage of unstimulated cells with the transcription factor NF-κB (p65) in the cytoplasm. After stimulation, treated cells have a significantly higher similarity score of NF-B (p65) with nuclear stain PI, indicating that NF-κB (p65) translocated into the nucleus after stimulation (median similarity scores are 1.52 and 3.01, respectively, **Fig. 2**). Similar results are noted in TLR5-stimulated monocytes[9].

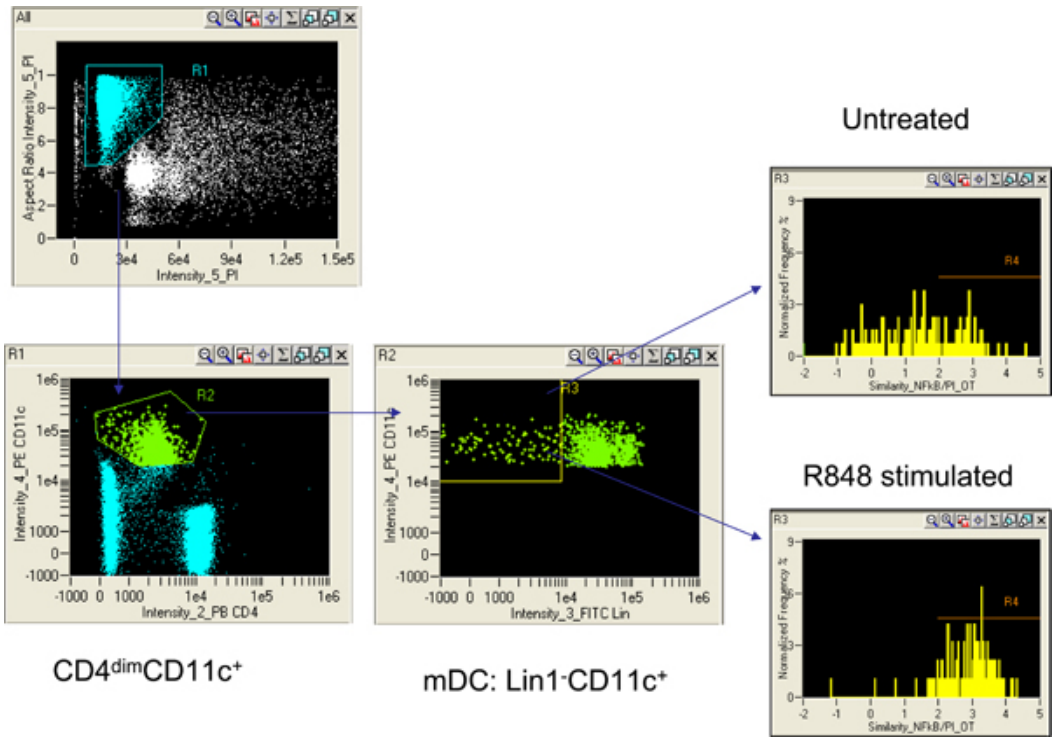

Figure 1. Staining and Gating Strategy to quantify effects of stimulation on human mDCs. The NF-κB transcription factor regulates expression of numerous immune system genes. This study measures the nuclear localization of NF-κB (p65) in mDCs from one representative

subject after TLR7/8 ligand stimulation. In-focus single cells were identified by gating on PI positive events with high nuclear aspect ratios (R1). mDCs (Lin1-, CD4dim, CD11c+) were gated in R3. Nuclear localization of NF-κB (p65) is plotted in R4. Click here to view larger figure.

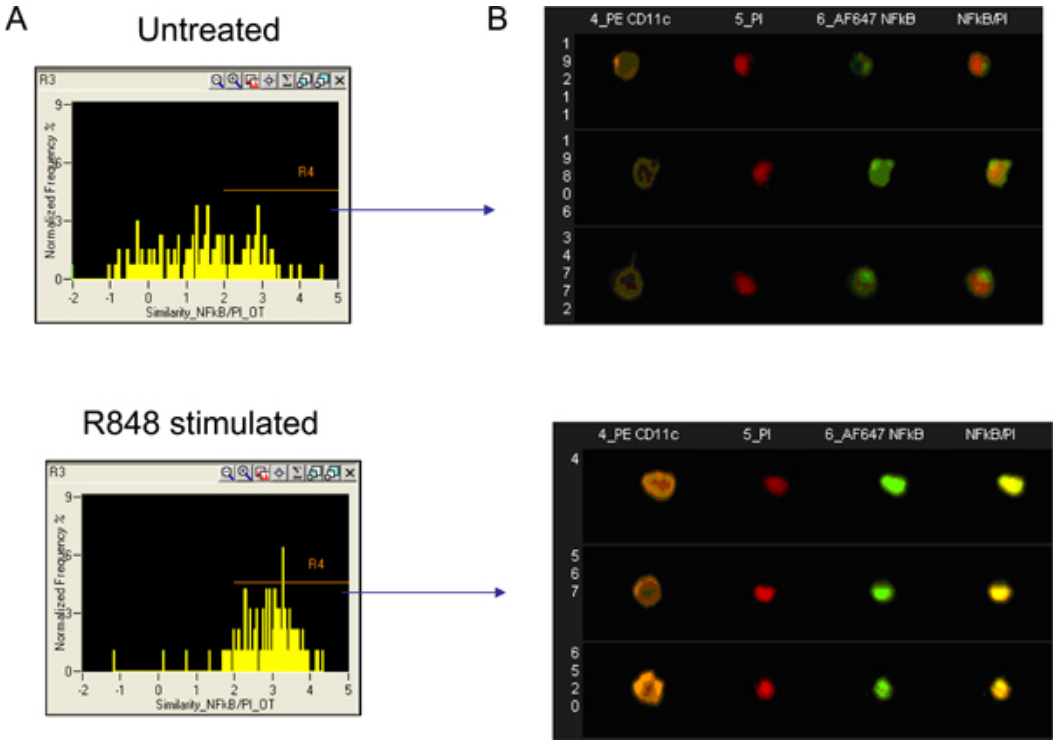

Figure 2. Translocation of NF-κB in mDCs after stimulation. The translocation of NF-κB (p65) into the nucleus after stimulation (R4) is depicted in populations of untreated and stimulated mDCs; the median similarity score is 1.52 in the untreated sample and 3.01 in the stimulated sample (A). Digital images collected simultaneously of the untreated or R848-stimulated cell populations show representative cells and the intensity of NF-B translocated into the cell nucleus (B). Click here to view larger figure.

Discussion

The critical steps in use of ImageStream for translational studies are the selection of relevant comparison groups and optimization and validation of antibody specificity. Differences between subject groups in the labeled target will be readily apparent through histograms and cell images. In combination with a thorough analysis of changes in relevant receptors and signaling pathways, these data will provide valuable insight into mechanisms that underlie immune dysregulation in specialized cohorts. The technique will be limited by availability of specific antibodies of sufficient affinity for relevant targets. In addition, the instrument is most suitable for a multi-user facility and the analysis software requires some care to master.

This technology is a powerful technique for translational investigations of human diseases. Advances in genome sequencing and array technology have allowed identification of variants implicated in many diseases, but rarely identify the mechanism of action of the nominated gene. Our studies provide a blueprint for translational investigations in individual subjects such as the nuclear to cytoplasmic ratio of NF-κB after stimulation. From a small sample of blood, we can identify differential processing or signaling in a lineage specific manner in a subject's own cells. Using this method, we have quantified cellular responses in monocytes of a large cohort of young and elderly subjects and demonstrated alterations in cell signaling in aging[9]. ImageStream may be applied more broadly to highlight key mechanisms in cells in many investigations such as therapy responders and non-responders, or for an individual subject before and after treatment, or during flare and remission. With abundant modifications possible for choice of subject groups under study, cell types, and cellular mechanisms to investigate, the future applications of ImageStream are numerous and should allow significant advances in many translational areas.

Disclosures

No conflicts of interest declared.

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