

A NOVEL METHOD TO ASSESS THE LEVEL OF IN VIVO IMMUNOSUPPRESSION IN TRANSPLANT PATIENTS USING AMNIS IMAGESTREAM TECHNOLOGY

Oleh Pankewycz¹, Hans Minderman², Paul Wallace², Lin Feng¹, Mark R. Laftavi¹.

¹Univ. At Buffalo/Kaleida Health Multiorgan Transplant Center, Buffalo General Hospital, Buffalo, NY 14203

²Flow and Image Cytometry Facility, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263

Introduction

Currently, immunosuppressive therapy is based on empiric drug dosing guidelines rather than on objective measures of the *in vivo* effectiveness of these agents. Given that the intracellular distribution of NF-κB reflects the activation state of immune cells, we measured the degree to which NF-κB translocation was impaired in peripheral blood T lymphocytes obtained from patients receiving conventional immunosuppressive therapy. Intracellular NF-κB localization was quantitatively analyzed in this heterogeneous cell population with the ImageStream platform, which combines the high throughput power of flow cytometry and the imaging information of microscopy.

Objectives

1. Establish that the ImageStream detection of NF-κB nuclear translocation reliably reflects human T cell activation events.
2. Determine if immunosuppressive therapy *in vivo* affects the ability of human T cells to translocate NF-κB when stimulated *in vitro* as measured by ImageStream analysis.
3. Determine if the degree of impaired NF-κB nuclear translocation varies amongst patients and correlates with intensity of *in vivo* immunosuppression or clinical outcomes.
4. Correlate ImageStream and ImmunoKnow (Cylex) results.

Materials and Methods

Patients:

- Peripheral blood leukocytes obtained from 8 healthy volunteers and 9 kidney transplant recipients treated with immunosuppressive therapy.
- Patients were treated with tacrolimus (n= 8) or sirolimus (n= 1), mycophenolic acid (n= 9) and prednisone
- Transplant recipients were 3 to 30 months post-transplant
- Stable patients (n=5) had no evidence of renal dysfunction or infection at the time of analysis
- Patients with rejection (n=2) had biopsy proven Grade 1A rejections at the time of or immediately prior to analysis
- Patients with infections (n=2) suffered from H. zoster or BK viral infections at the time of analysis.

NF-κB Activation:

- NF-κB translocation was induced by exposure to PMA (10 ng/ml) and ionomycin (1.5 μM) for 24 hours

Antibodies and Nuclear Staining:

- PE-conjugated anti-CD3, anti-CD4 and anti-CD8 antibodies
- FITC conjugated anti-p65 antibody (SantaCruz Biotechnology Inc, Santa Cruz, CA)
- DRAQ5 nuclear stain (Biostatus Limited, Leicestershire, United Kingdom)

Materials and Methods, cont'd

Briefly, one green top of heparanized blood was obtained for each sample. Lymphocytes were enriched by Ficoll density gradient. These were then washed, counted, resuspended in RPMI 1640 media supplemented with 20% FBS, and allowed to incubate overnight at 37 degrees.

The following morning, each sample was split into two, one half in media, the other in media containing PMA and Ionomycin, and incubated for 1 hour at 37 degrees. After washing, the samples were then split into tubes according to the conditions below, normal mouse IgG added (for blocking of non specific binding), and allowed to sit on ice for 10 minutes, before adding the respective antibody.

- (1) Unstimulated w CD4-PE
- (2) Unstimulated w CD8-PE
- (3) Unstimulated w CD3-PE
- (4) Stimulated w CD4-PE
- (5) Stimulated w CD8-PE
- (6) Stimulated w CD3-PE

Following surface staining, and subsequent washing, each sample was fixed in 4% Para formaldehyde (PFA) and stained with NFκB. Prior to running on the Imagestream, each sample was stained with the nuclear dye DRAQ5.

Data acquisition was performed with the ImageStream platform (Amnis Corp., Seattle, WA) which captures high-resolution images of cells in flow at rates of up to 100 cells per second, effectively combining the imaging capabilities of microscopy with the high throughput power of flow cytometry.

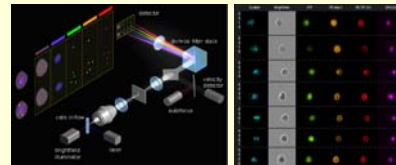
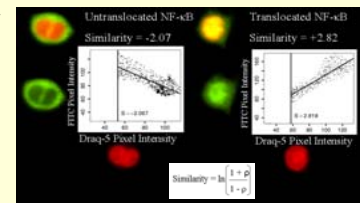
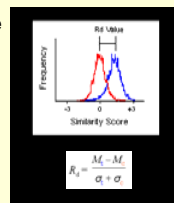


Image analysis algorithms to quantify NF-κB translocation events. The IDEAS® image analysis software applies features (algorithms) and masking operations (region-finders), to perform image-based analysis. To assess events of translocation, a similarity score algorithm is used. This is calculated by log transforming the Pearson's correlation coefficient for pixel values of the DRAQ5 (nuclear) and FITC (NF-κB) signals. Changes in the similarity score distributions are reported as an Rd metric, which is a measure for the shift of two distributions (Fisher's Discriminant Ratio).

Similarity Score



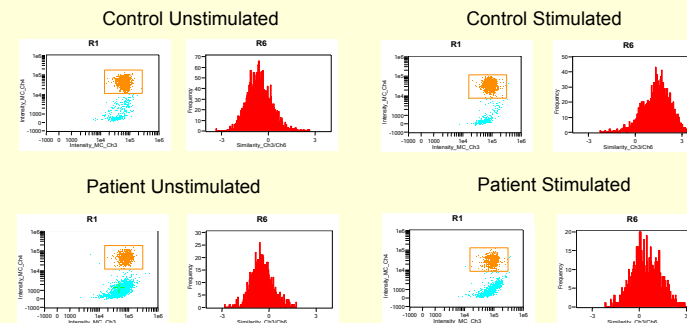
Rd Value



Results, cont'd

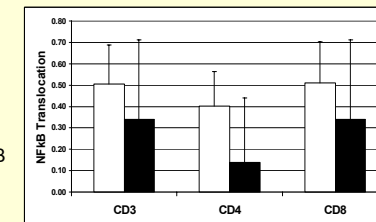
1. Example of Amnis ImageStream Analysis of PMA/Ionomycin Induced NF-κB Translocation in CD3 T cells

The following figures show the gating and staining for one individual sample. Note that R1 represents double positive (CD3 marker + NF-κB) cells and R6 the similarity score of NfκB and Draq5 (nuclear stain) of the double positive cells in focus.



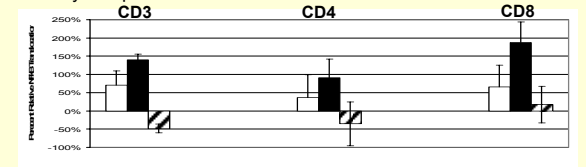
2. Amnis ImageStream Detects Decreased NF-κB Translocation In T Cells Obtained From Transplant Recipients.

Subsets of PBL isolated from normal controls (open bars, n=8) and from transplant recipients on immunosuppressive therapy (closed bars, n=9) were subjected to Amnis flow cytometric analysis for NFκB translocation following 24 hours of stimulation with PMA/ionomycin. NFκB translocation was diminished in all T cell subsets; however, only in CD4 T cells the results were significant with p<0.04.



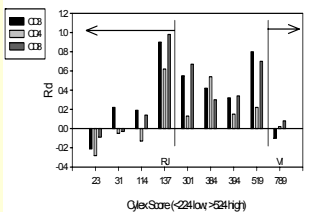
3. Relative NF-κB Translocation In T Cell Subsets in Transplant Recipients.

The results of four different assays are presented as the relative amount of nuclear NF-κB translocation in transplanted patients compared to normal controls. In each assay, the control patients were considered to have 100% NF-κB translocation. Compared to control patients in assays performed at the same time, 5 stable patients (open bars) had between 36 and 75% suppression of NFκB translocation all T cell subsets. In contrast, 2 patients with rejection (closed bars) had increased NFκB translocation in the CD3 and CD8 subsets and only minimal suppression in the CD4 subset (9%). Two patients (hatched bars) who had viral infections showed severely compromised NFκB translocation in all subsets.



4. Comparison of Amnis ImageStream and Cylex ImmuKnow Assays

In 7 of the 9 patients tested, there was a general correlation between both assays. However, in 2 patients the Amnis ImageStream analysis better reflected clinical circumstances. A patient with rejection (RJ) had a low Cylex score suggesting over-immunosuppression yet showed little NF-κB inhibition in the ImageStream assay consistent with under-immunosuppression. Another patient (VI) had H. zoster infection despite having a very high Cylex score suggesting immunocompetence while the ability to translocate NF-κB was severely impaired.



Conclusions

- ImageStream analysis allows quantitative assessment of differences in NF-κB translocation events in stimulated versus unstimulated human T cells.
- Immunosuppressive therapy *in vivo* leads to diminished NF-κB responsiveness *in vitro* that can be measured by the ImageStream Assay.
- The level of NF-κB impaired translocation *in vitro* correlates with the degree of therapeutic immunosuppression achieved *in vivo*.
- ImageStream analysis should prove to be extremely useful to determine NF-κB activity as a parameter of response in (immunophenotypically definable) target cells in order to guide individualized immunosuppression.