NIH Public Access
Author Manuscript
Transplant Proc. Author manuscript; available in PMC 2013 September 19.

Published in final edited form as:
Transplant Proc. 2009 ; 41(1): 238–239. doi:10.1016/j.transproceed.2008.10.060.

ITSC08: *IN SITU* QUANTITATIVE IMMUNOPROFILING OF REGULATORY T CELLS USING LASER SCANNING CYTOMETRY

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Abstract

Laser scanning cytometry (iCys; CompuCyte) is recently developed methodology that utilizes fluorescence-based quantitative measurements on tissue sections or other cellular preparations at a single-cell level. The purpose of this study was to develop objective quantitative immunoprofiling of regulatory T cells (T regs) on formalin-fixed/paraffin embedded (FFPE) biopsy sample from transplanted allograft using iCys. We analyzed the population of CD4 (+) Foxp3 (+) T regs among the entire CD4 (+) T cells and the entire T cells (the total of CD4 (+) and CD8 (+)) in human intestinal allograft biopsy samples to evaluate the usefulness of iCys. Primary antibodies (Foxp3 and CD4) were incubated on one section. Foxp3 and CD4 were labeled by Alexa Fluoro 488 (Alexa488) and 647 (Alexa647) using polymer HRP and catalyzed signal amplification. On the other section, CD8 and CD4 were labeled by Alexa488 and Alexa647 using the same protocol. Data acquisition was performed using iCys. The signal intensity of Alexa488 and Alexa647 was sufficient to analyze by iCys. Distribution of the integrals of Alexa488 and Alexa647 visualize each cell population and enable to calculate the population of T reg among CD4 (+) T cell, CD4 (+) T cell among the total T cells and T reg among the entire T cells. iCys and signal amplified immunofluorescent staining allowed objective quantitative immunoprofiling of *in situ* T reg populations, with precise quantitative analysis at a single-cell level on FFPE section. This objective method can be applied on the biopsy sample from various transplant organs.

Introduction

Cytofluorographic analysis enables to analyze suspended cell populations quantitatively. On the contrary, quantitative *in situ* analysis (i.e. in a tissue sections) is difficult because of a lack of objective methodology, and the immunophenotypic evaluation of local inflammatory infiltrate is performed with semi-quantitative method (*1, 2*). However, the semi-quantitative method is always accompanied by the issues of reproducibility (*1, 3, 4*). Laser scanning cytometry (iCys; CompuCyte) is recently developed methodology that utilizes fluorescence-based quantitative measurements on tissue sections or other cellular preparations at a single-cell level (*5*). The purpose of this study was to develop objective quantitative immunoprofiling of regulatory T cells (T regs) on formalin-fixed/paraffin embedded (FFPE) biopsy sample from transplanted allograft using iCys.

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Methods

We analyzed the population of CD4 (+) Foxp3 (+) T regs among the entire CD4 (+) T cells and the entire T cells (the total of CD4 (+) and CD8 (+)) in human intestinal allograft biopsy samples to evaluate the usefulness of iCys. Two unstained sections of four μm in thickness were prepared from the routinely processed FFPE tissue. After deparaffinization and blocking, primary antibodies (Foxp3 and CD4) were incubated on one section. Foxp3 and CD4 were labeled by Alexa Fluoro 488 (Alexa488) and 647 (Alexa647) using polymer HRP and catalyzed signal amplification. Propidium iodide (PI) counter staining was performed to visualize nuclear. On the other section, CD8 and CD4 were labeled by Alexa488 and Alexa647 using the same protocol. Data acquisition was performed using iCys. Determination of nucleated cells was achieved by recognizing PI signals. The following factors were recorded: area, x position, y position, fluorescence integral and maximum intensity for all channels. Alexa488 (Foxp3 or CD8) and Alexa647 (CD4) integrals in section were acquired at a single-cell level. Each Foxp3 or CD8 positive event was recorded utilizing the argon laser and green detector. Each CD4 positive event was recorded utilizing the HeNe laser and red detector. Single cell population was identified and gated according to the PI positive area. Further assessments were performed only on the single cell population. The each cell population was calculated based on the distribution of the integrals of Alexa488 and Alexa647 on each slide.

Results

An example of intestinal allograft biopsy which histopathologically showed indeterminate for acute rejection with mild lymphoplasmacyitc infiltrate was evaluated. The nuclear signal of each cell was well determined by the PI signal. The signal intensity of Alexa488 and Alexa647 were sufficient to analyze by iCys. Distribution of the integrals of Alexa488 and Alexa647 on the section in which Foxp3 and CD4 were labeled was visualized and CD4 (+) cell population was gated. The population of T reg (Foxp3 (+) and CD 4(+) cell) among CD4 (+) T cell was 13.7%. Distribution of the integrals of Alexa488 and Alexa647 on the section in which CD8 and CD4 were labeled was visualized. The population of CD4 (+) T cell among the entire T cells (the total of CD4 (+) and CD8 (+) T cells) was 20.9%. Accordingly, the population of T reg among the entire T cells was 2.9%.

Conclusion

iCys and signal amplified immunofluorescent staining allowed objective quantitative immunoprofiling of *in situ* T reg populations, with precise quantitative analysis at a single-cell level on FPE section. Combined analysis of various immunoprofiling enables to investigate more detailed analysis on the *in situ* T reg profiling. This objective method can be applied on the biopsy sample from various transplant organs.

Acknowledgments

This work was partially supported by NIH-NCRR, GCRC MO1RR16587, NIDDK RO1-DK55347-IU42RR016603, 5RO1 DK25802, ICR 5U42RR016603, JDRFI 4-200-946 and 4-2004-361, and the Diabetes Research Institute Foundation.

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