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Effect of Cell Density and HLA-DR Incompatibility on T-Cell Proliferation and Forkhead Box P3 Expression in Human Mixed Lymphocyte Reaction

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ABSTRACT

Background. The proliferation rates of human T cells in vitro are affected by some factors such as initial T-cell number, dose of stimulating cells, and duration of culture. The transcription factor forkhead box P3 (FoxP3) has been used to identify regulatory T cells in humans and is thought to correlate with tolerance to allogeneic organ transplant. Thus, it is important to optimize conditions to expand FoxP3 cell proliferation to improve engraftment of allogeneic organ transplants.

Methods. We studied proliferative responses and FoxP3 expression in divided T cells with the use of flow cytometric analysis of Ki-67 in culture of different concentrations of responding cells (6×10^6, 4×10^6, 2×10^6, 1×10^6, and 0.5×10^6cells/mL), different types of stimulating cells (lymphocytes and low density cells), and different numbers of HLA mismatches.

Results. The proportion of CD3(+) cells, CD4(+)CD25(+) cells, and CD4(+)CD25(+) FoxP3(+) cells among mononuclear cells were highest at initial cell concentration of 2×10^6 responder cells/mL with lymphocytes as stimulators at day-5 mixed lymphocyte reaction (MLR). They were highest at a concentration of 4×10^6 responder cells/mL with low density cells as stimulators. The recovery (%), proportion of CD3(+) cells, CD4(+) CD25(+) cells, and CD4(+)CD25(+)FoxP3(+) cells with 2 HLA-DR incompatibility were significantly higher than those of 1 HLA-DR incompatibility at day-5 MLR.

Conclusions. Initial cell concentration and HLA-DR incompatibility can affect the generation of FoxP3+ T cells in human MLR. These factors could be considered for efficient generation of Tregs for clinical trials in the future.

THE DETECTION of the intracellular transcription factor forkhead box P3 (FoxP3) in experimental animals has been associated with functional regulatory T cells (Tregs) [1]. In humans, FoxP3 can mark activated cells, but sustained expression of FoxP3 is still the most reliable marker for human Tregs [2]. The generation of T cells in vitro has been known to depend on many factors such as concentration of responding cells and stimulating cells, ratio of responding cells to stimulator cells [3], type of simulating cells, duration of culture, and degree of HLA mismatches [4]. However, little is known about the generation of human Tregs in vitro in different culture conditions for transplantation recipients. The aim of this study was to reveal the effects of concentration of responding cells and HLA incompatibility on the generation of FoxP3+ T cells in human mixed lymphocyte reaction (MLR).

MATERIALS AND METHODS

MLR Responder and Stimulator Cells

The residual peripheral blood samples of 13 patients awaiting kidney transplantation or allogeneic hematopoietic stem cell transplantation

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and 1 healthy volunteer were used within 24 hours of blood sampling. The recipients/donor combinations were randomly selected. HLA types for HLA-A, -B, -C, -DRB1, and -DQB1 loci were typed by the UCSD Immunogenetics and Transplant Laboratory with the use of LABType SSO (One Lambda, Inc, Canoga Park, California, United States) or by sequence-based typing by use of Histogenetics (Ossining, New York, United States). The research was approved by the University of San Diego California Institutional Review Board.

Peripheral Blood Mononuclear Cell Isolation, Preparation of Antigen-Presenting Cells, and MLR

Peripheral blood mononuclear cells were separated through the use of lymphocyte separation media (Mediatech, Inc, Manassas, Virginia, United States) by means of the density gradient method. AIM-V (Gibco Life Technologies, Gaithersburg, Maryland, United States) media was used for entire culture procedures. Stimulating cells were treated with mitomycin C (Accord Healthcare, Durham, North Carolina, United States) in a final concentration of 25 μg/mL solution for at least 30 minutes at 37°C in a 5% CO_2 incubator and washed 3 times as previously reported [5]. Cultures were incubated at 37°C in a 5% CO_2 incubator for 5 days. Lymphocyte fraction was separated by use of the plastic adherence method [6], and low-density cells (LDC), containing dendritic cells and macrophages, were prepared after overnight incubation as previously reported [7]. To see the effect of different concentrations of responding cells and stimulator cells, 5 different concentrations of responder cells and stimulator cells (6×10^6, 4×10^6, 2×10^6, 1×10^6, and 0.5×10^6 cells/mL) were used for each of 2 different types of stimulating cells (lymphocytes and LDCs). Cells were incubated at 37°C in a 5% CO_2 incubator for 5 days.

Flow Cytometry Analysis

Cells were tested at 5 days in culture and were stained with antigen-presenting cell (APC)-Cy7–conjugated anti-human CD3, fluorescein isothiocyanate–CD4, and phycoerythrin (PE)-Cy7 CD25. For intracellular staining, cells were then fixed and permeabilized by use of the Human FoxP3 Buffer Set (BD Biosciences, San Jose, California, United States). Nonspecific binding was blocked by means of incubation in 10% normal mouse serum at room temperature for 20 minutes and stained with APC-conjugated anti-human FoxP3 and PE-conjugated anti-human Ki-67 (Biolegend, San Diego, California, United States). Cells were washed twice and read with the use of the FACSCanto II flow cytometer (BD Biosciences). Isotype controls were used to determine non-specific staining (Fig 1).

Cytokine Measurement

Culture supernatants were collected at day 5 and stored at −70°C until analysis. The Human Th1/Th2 11plex Ready-to-Use FlowCytomix Multiplex kit (eBiosciences, San Diego, California, United States) was used to measure the concentrations of human interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, interferon-γ, tumor necrosis factor (TNF)-α, and TNF-β according to the manufacturer's instructions.

Statistical Analysis

Categorical variables were compared by means of χ^2 test or Fischer's exact test where appropriate. Non-categorical variables were analyzed by means of analysis of variance, Student *t* test, or Wilcoxon signed rank test where appropriate. Statistical significance was set at a 2-sided α level of .05. All analysis was performed with the use of SPSS version 12.0 (IBM Inc, Armonk, New York, United States).

RESULTS

As described in the Materials and Methods section, standard MLR cultures were performed with the use of stimulator cells from patient samples received in the UCSD transplant laboratory and responder cells from a normal donor. Various cell concentrations were used to determine the optimal recovery of cells after 5 and 7 days of culture.

Effect of Cell Concentrations of Responder Cells on Recovery

The recovery (%) at day-5 MLR was higher according to the initial number of responder cells (Fig 2). The recovery (%) at concentration of 6×10^6/mL responder cells was significantly higher than that at concentrations of 2×10^6/mL ($P = .008$), 1×10^6/mL ($P = .003$), or 0.5×10^6/mL ($P = .014$). The recovery (%) at a concentration of 4×10^6/mL responder cells was significantly higher than that at a concentration of 1×10^6/mL cells ($P = .031$).

Effects of Cell Concentrations of Responder Cells on Cell Differentiation of MLR With the Use of Lymphocytes as Stimulator Cells

The proportion of CD3(+) cells among mononuclear cells at day-5 MLR with the use of lymphocytes as stimulator cells was different according to the cell concentrations of responder cells (Fig 3A). The proportion of CD3(+) cells at a concentration of 6×10^5 responder cells was significantly higher than that at a concentration of 4×10^6/mL ($P = .043$) or showed a tendency of increase compared with that at 2×10^5 cells ($P = .063$). The proportion of CD4(+)25(+) cells among mononuclear cells at day-5 MLR with the use of lymphocytes as stimulator cells was different according to the cell concentrations of responder cells. The proportion of CD4(+)25(+) cells at a concentration of 2×10^6/mL responder cells was significantly higher than that at a concentration of 1×10^6/mL ($P = .018$) or 6×10^6/mL ($P = .021$). The proportion of CD4(+)25(+) cells at a concentration of 4×10^6/mL responder cells was significantly higher than that at concentration of 6×10^6/mL ($P = .017$).

The proportion of CD4(+)25(+)FoxP3(+) cells at a concentration of 4×10^6/mL responder cells was significantly higher than that at concentrations of 1×10^6/mL ($P = .046$), 2×10^6/mL ($P = .049$), or 6×10^6/mL ($P = .046$). The proportion of CD4(+)25(+)FoxP3(+) cells at a concentration of 2×10^6/mL responder cells was significantly higher than that at a concentration of 1×10^6/mL ($P = .042$). The ratio of proliferating Tregs to non-proliferating Tregs was highest at a concentration of 4×10^6/mL responder cells and significantly higher than that at a concentration of 6×10^6/mL ($P = .036$) or showed a tendency of increase compared with that at 2×10^6/mL ($P = .066$) (Fig 3A).

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