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Characterization of CD25+ T regulatory cells in Systemic Lupus Erythematosus

Bryan Lucore

ABSTRACT

Very recently suppressor T cells have been shown to function as regulatory T or T regulatory (TR) cells. TR cells are subdivided into TR induced and TR naturally occurring such CD4+CD25+ (Thymic derived, TR), and natural killer cells (NKC). Systemic Lupus Erythematosus (SLE), a prototypical systemic autoimmune disease, is immunologically characterized by the loss of self-tolerance to a myriad of autoantigens. We set out to characterize the TR cells in SLE, both quantitatively and in their regulatory capability. We have developed an isolation procedure for TR from peripheral blood of SLE patients and age matched normal controls. The first step consists of a magnetically CD4+ enrichment followed by cell sorting in a FACSDiva for CD4+ single positive and CD4+CD25+ TR. We consistently obtained a purity of greater than 90% for both subsets. Our preliminary analysis revealed that quiescent lupus patients, i.e. SLEDAI _ 2 (SLE disease activity index) have on average an increased percentage (17%) of CD4+CD25+ as compared to normal controls (7.5-10%), but active lupus patents (SLEDAI > 4) have up to a 17% increase in CD4+CD25+ TR. Phenotypically CD4+CD25+ TR from active lupus showed up to a 50% higher proportion of GITR (glucocorticoid-induced Tumor Necrosis Factor receptor) expression as compared with CD4+CD25+ from normals that expressed it in 30%. Preliminary results assessing the regulatory function of TR *in vitro* showed a lack of suppression by CD4+CD25+ from Lupus patients. We have embarked on a series of experiments to establish the therapeutic potential of manipulating TR function in Lupus.

Keywords: *autoimmunity, CD4+, CD25+, CD4+CD25+, Systemic Lupus Erythematosus, T lymphocytes*

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease that is the result of a series of interactions within the immune system that ultimately lead to the loss of self-tolerance to nuclear autoantigens. In SLE, T- and B- cell autoimmune responses result in the generation of autoantibodies and immunecomplexes, along with autoreactive T cells, which together cause pathology in several target organs, including the joints, skin, kidneys, heart, lungs, blood vessels, and the brain (Novak, 1997; Solomou et al., 2001). SLE can occur in any sex, race, or stage of life, but usually surfaces in minorities, females, and around the ages of 15 to 45 years of age (Alarcon et al., 1999; Novak, 1997). The cause of lupus is currently unknown, although it could occur from genetic, environmental, and possibly hormonal factors. Current research is suggesting that genetics may play a role in lupus (Novak, 1997).

The three main functions that the T lymphocytes carry out in immune response are: to kill pathogen infected cells and make them inaccessible to antibodies, to maintain the inflammatory response at infections sites, and to regulate adaptive and innate features of the immune responses. The regulatory functions of T lymphocytes are carried out by helper T cells (CD4), which regulate the immune responses (Basiro, 1990). TR cells can mediate their effector function via multiple mechanisms, some of which are unknown. The control of pathogenic immune responses by TR cells has different phases. In the steady state, low numbers of immature dendritic cells (DC) move to the draining lymph node (LN) from uninflamed tissues and present self-peptides to both TR and T path, which, due to intrinsically high affinity of the TR cells, leads to low-level activation of TR and inhibition of T path (Banchereau and Steinman, 1998). During immune response high numbers of activated mature dendritic cells (mDC) move to the LN where they present peptides derived from both self and foreign antigens. After the infectious agent is cleared, effector T (Te) cells either die or become memory T cells. The regulatory phase is characterized by presentation of self-peptides.

The goal of this project is to characterize the TR cells in SLE, both quantitatively and their regulatory capability. The ability to induce or proliferate TR cells *in vivo* and *in vitro* could have important implications not only in the field of autoimmunity, but also in transplantation tolerance. An important advantage is that, because TR cells can exert bystander suppression in a nonantigen-specific manner, they need not necessarily recognize the target antigen(s) that are the subject of immune attack (Check, 2002). Induction of TR cells that react to any local tissue-expressed molecule may be sufficient to inhibit immune pathology. Identification of the sites of action of TR cells and of their antigen reactivities will be paramount in applying this kind of strategy to the treatment of inflammatory diseases. One important area that remains to be addressed is whether TR cells can also down-regulate ongoing immunopathological reactions such as an established clinical autoimmune disease. If

so, what manipulations are required to re-establish dominant T reg cell activity *in vivo*? In addition, identification of downstream cellular targets and molecular mechanisms of T reg cell action should further enhance the development of treatments that inhibit immune pathology. Manipulation of T reg cells may also have important clinical benefits in the induction of protective immunity.

MATERIALS AND METHODS

Lymphocyte Isolation

Samples of heparinized venous blood cells were obtained from healthy controls and from patients of The National Institute of Arthritis, Musculoskeletal and Skin Diseases (NIAMS), a component of the National Institutes of Health, Bethesda, MD. The peripheral blood mononuclear cell samples were separated from the red blood cells by Ficoll-Paque density gradient and centrifuged at 20^{o}C for 25 minutes at 600g. The lymphocytes were then removed and washed three times with Dulbecco's phosphate buffered saline (DPBS, Sigma, St. Louis, MO) and counted in a Hausser scientific hemocytometer with trypan blue stain (Bio-Whittaker; Walkersville, MD). The lymphocytes were then enriched with CD4+ magnetic microbeads and sorted into CD4+ and CD25+ cells with an autoMACS (Miltenyi Biotec, Auburn, CA).

T Cell Culture

The isolated CD 4+ and CD 25+ cells were then separately suspended into T cell medium (TCM) containing RPMI-1640 with L-glutamine (Gibco, Grand Island, NY), 10% FCS (Hyclone, Logan, UT), 55µM 2-mercaptoethanol (Gibco), 10 mM HEPES (Gibco), 2.5 ml nonessential amino acids (Gibco), 2.5 ml essential amino acids (Gibco), and 20µg/ml Gentamicin (Gibco). The suspension was then plated into 24 well tissue culture plates at 1ml per well. The plates were then placed into a 37^{o}C incubator with 5%CO_2. After four days, half of the medium from each well was removed with a sterile pipette and replaced with an equal volume of fresh TCM with IL-2. This was repeated every three days or more often it the growth was vigorous and the medium turned yellow. Starting the fifth day the wells were inspected under a microscope. If large numbers of blasts were present and the cells appeared to be crowded, the wells were split and resuspended by removing half of the medium with a Pasteur pipette and transferring to another well in a fresh 24 well plate.

Cell Sorting

The cultured cells were then removed and centrifuged down at 1500 RPM and resuspended in Dulbecco's phosphate buffered saline. The cells were then stained with combinations of anti-CD4-Cyc, CD25-PE, and Isotype controls. After staining, the cells were sorted in a FACSDiva by their forward and side scatter properties. When sorting was completed the cells were run through a FACSCalibur to test their percent purity and then counted with a Hausser scientific hemocytometer with trypan blue stain. The cells were then cultured in a 24 well plate with TCM for 72 hours at 37^{o}C/5% CO_2.

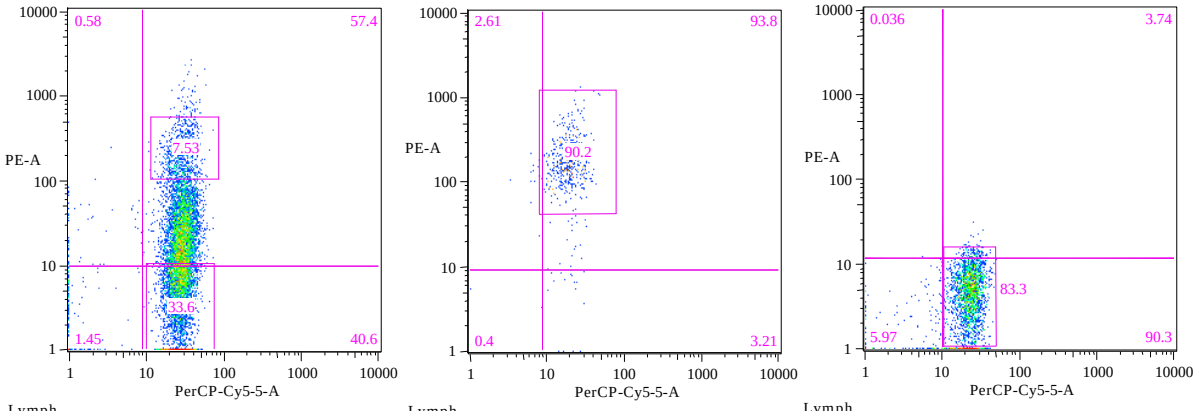
T cell proliferation assay

After the 72-hour culture, the cells were transferred to a 96 well microtiter plate and 50 µl 1 µCi/ml [^{3} H] thymidine was added to each well. The plates were cultured for an additional 16 hours and then harvested with a scintillation counter, which measured the [^{3} H] thymidine uptake of the cells.

RESULTS

Cell Sorting

Representative data showing the percentages of cells of each subset obtained are presented in Figure 1. The purity contents are boxed and labeled with the percentage.



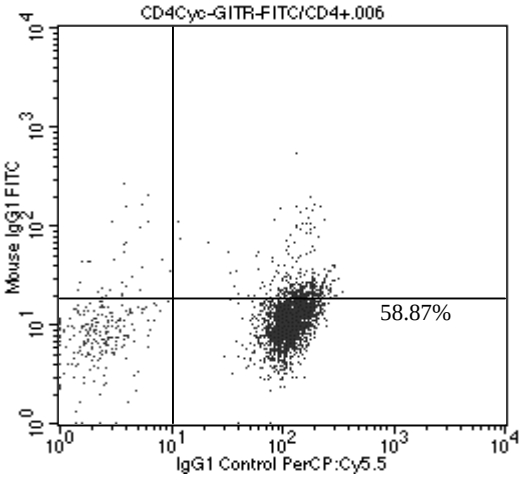


Figure 1. Representative cell-sorting of peripheral blood mononuclear cells from SLE patient in remission. Glucocorticoid-induced TNF receptor (GITR) expression in a healthy control is shown above.

T Cell Proliferation

Purified CD4+ (100,000 cells per well) were tested for their ability to proliferate in response to allogenic dendritic cells. The data are shown in Figure 2.

DISCUSSION

Cell sorting the CD4+CD25+ TR cells consistently obtained a purity greater than 90% for both CD4+ single positive and CD4+CD25+. Analysis revealed that inactive lupus patients, i.e. SLEDAI _ 2 have on average an increased percentage (17%) of CD4+CD25+ as compared to normal controls (7.5-10%), but active lupus patients (SLEDAI > 4) have a substantial increase up to 17% in CD4+CD25+ TR. Phenotypically CD4+CD25+ TR cells from active lupus patients showed a higher proportion of GITR expression up to 50% as compared with CD4+CD25+ from normal controls that expressed it in 30%. Results assessing the regulatory function of TR cells
in vitro showed a lack of suppression by CD4+CD25+ from Lupus patients.

The T cell proliferation assay, as represented in Figure 2, of the CD25+CD4+ TR cells constituted about 10% of the peripheral CD4+ T cells and possessed potent regulatory activity *in vitro*. They show a partially anergic phenotype, in that they proliferate poorly upon T cell antigen receptor (TCR) stimulation *in vitro* and their growth is dependent on exogenous IL-2. Active SLE patients were found to have a normal percentage of CD25+CD4+ TR cells while SLE patients in remission showed an increased proportion. Regarding the phenotype, preliminary results showed a higher proportion of CD25+ TR cells. More importantly, CD25+ TR cells from lupus patients in remission were incapable of suppressing the proliferated response of CD4+ effectors to alloantigens, this defect was more profound when CD25+ TR cells from active lupus patients were tested. The mechanisms of CD25+ TR regulatory function are unclear at the present time but require cell contact (McHugh et al., 2001). *In vitro* suppression requires activation of TR cells via their TCR, does not involve killing of the responder cells and is mediated through a cell-contact dependent mechanism independent of IL-4, IL-10, and TGFβ. The mechanism causing the defect in suppression of

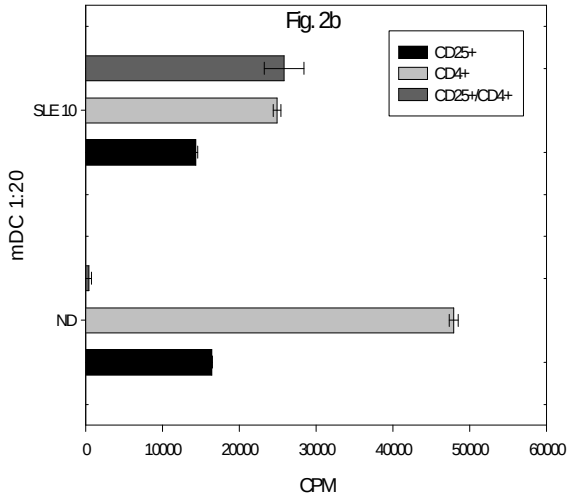


Figure 2. T cell proliferation results for SLE patients after 72 hour [^{3} H] culture. SLE patient in remission shown in graph (A) and active SLE patient shown in graph (B).

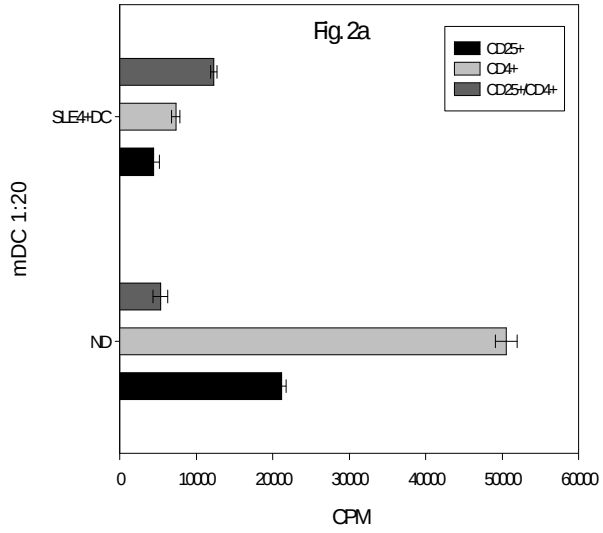


Table 1. Percentages obtained from sorting the peripheral blood mononuclear cells.

Sample Type	CD4+CD25+ Percentage
Normal Donor	7.5-10%
SLE in Remission	17%
Active SLE	27%

the lupus TR cells remains to be determined and the elucidation of this as well as the ability to isolate and manipulate these cells offers to expedite progress towards clinical use of TR cells as a cellular therapy for autoimmune diseases such as Systemic Lupus Erythematosus.

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