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Advancements on phenotypic and functional characterization of non–antigen-specific CD8+CD28− regulatory T cells

Daniela Fenoglio [a,b], Francesca Ferrera [a], Marco Fravega [a], Piercesare Balestra [a], Florinda Battaglia [a], Michele Proietti [a,b], Cristina Andrei [a,b], Daniel Olive [c], La Cava Antonio [d], Francesco Indiveri [a,b], Gilberto Filaci [a,b,*]

[a] *Centre of Excellence for Biomedical Research, University of Genoa, Genoa, Italy*
[b] *Department of Internal Medicine, University of Genoa, Genoa, Italy*
[c] *Laboratoire d'Immunologie des Tumeurs, INSERM UMR 599, Institut Paoli-Calmettes, Université de la Mediterranée, Marseille, France*
[d] *Department of Medicine, David Geffen School of Medicine, University of California–Los Angeles, Los Angeles, CA, USA*

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ABSTRACT

Among the different regulatory T lymphocyte (Treg) subpopulations, non–antigen-specific CD8+CD28− Treg (CD8+CD28− Treg) have been characterized for being involved in the pathogenesis of autoimmune diseases and cancer. A better phenotypic and functional characterization of this regulatory T-cell subset could help in identifying modulators of their activity with therapeutic finalities. The results of the present work show that Foxp3, a transcriptional marker of natural CD4+CD25+ Treg, is not expressed by CD8+CD28− Treg, thus indicating different origin and pathways of function for the latter with respect to the former regulatory cell type. Moreover, the results underline that the glucocorticoid induced TNF receptor is involved in generation processes but not in suppressor function of CD8+CD28− Treg. Phenotypic analyses demonstrate that, during their commitment from circulating nonregulatory CD8+CD28− T lymphocytes to Treg (an interleukin-10–dependent process), these cells downmodulate the IL7-receptor, thus differentiating them from long-lived, memory CD8+ T lymphocytes. Interestingly, CD8+CD28− Treg have been found to be resistant to the inhibitory effects of methylprednisolone, one of the most frequently administered corticosteroid drug used in therapy for immunosuppressive purposes.

1. Introduction

Studies performed in the last decade clearly revealed the main role played by regulatory T lymphocytes (Treg) in maintaining immune homeostasis at the periphery [1,2]. Indeed, great efforts have been made in several laboratories throughout the world to characterize cell types and identify functions of Treg in both healthy and pathologic conditions. Unfortunately, the increase of knowledge on Treg has not been associated with a better understanding of their functional network as well as of their pathogenic relevance in different diseases because of the high level of complexity of the regulatory circuits. Several Treg subtypes have been described within each of the two main subcategories, CD4+ Treg and CD8+ Treg [3,4]. At the moment it is really hard to define which of these Treg subpopulations has real relevance in specific physiologic or pathologic conditions. Similarly, it is not clear whether there is a functional hierarchy among Treg subpopulations as well as whether the Treg subtypes, separately classified according to actual knowledge, instead constitute unique subsets with variegate functional expressivity. Our studies in the recent years have been focused on CD8+ Treg and, in particular, on a subpopulation, constituted by non–antigen-specific CD8+CD28− Treg (CD8+CD28− Treg) [5], the pathogenic involvement of which in diseases has been strongly suggested by *ex vivo* analyses performed on cells purified from human specimens [6,7]. This CD8+CD28− Treg subset markedly differs from the antigen-specific CD8+CD28− Treg subpopulation, concerning both phenotypic and functional aspects [8,9].

Regarding the CD8+CD28− Treg subset several questions remain unanswered related to the expression of specific surface markers, the characterization of molecular pathways involved in generation and activation, the reason for their anergic status *in vitro* and the possible existence of modulators. The present manuscript will show the advancements recently achieved in our laboratory on phenotypic and functional characterization of CD8+CD28− Treg.

2. Subjects and methods

2.1. Generation of CD8+CD28− Treg

CD8+CD28− Treg were generated as described [10]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll-Hypaque gradient for 30 minutes at 1800

* Corresponding author.
E-mail address: gfilaci@unige.it (G. Filaci).

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rpm. PBMC were incubated in RPMI 1640 culture medium (Sigma, St. Louis, MO) added with 10% fetal calf serum (Sigma), 2% glutamine (Sigma) and penicillin 100 U/ml-streptomycine 0.1 mg/ml (Sigma) in culture flasks (Corning Costar, Cambridge, MA) at 37°C overnight. CD8+ T lymphocytes were purified from non adherent cells by magnetic beads separation. To positively select CD8+ T lymphocytes, magnetic beads coated with anti-CD8 monoclonal antibody (Dynabeads M-450 CD8, Invitrogen Dynal AS, Oslo, Norway) and the CD8 Detachbead (Invitrogen Dynal AS) were used according to the manufacturer's instructions. The positive selection procedure was repeated until the cell population was highly enriched in CD8+ T cells (>95%) as demonstrated by flow-cytometric

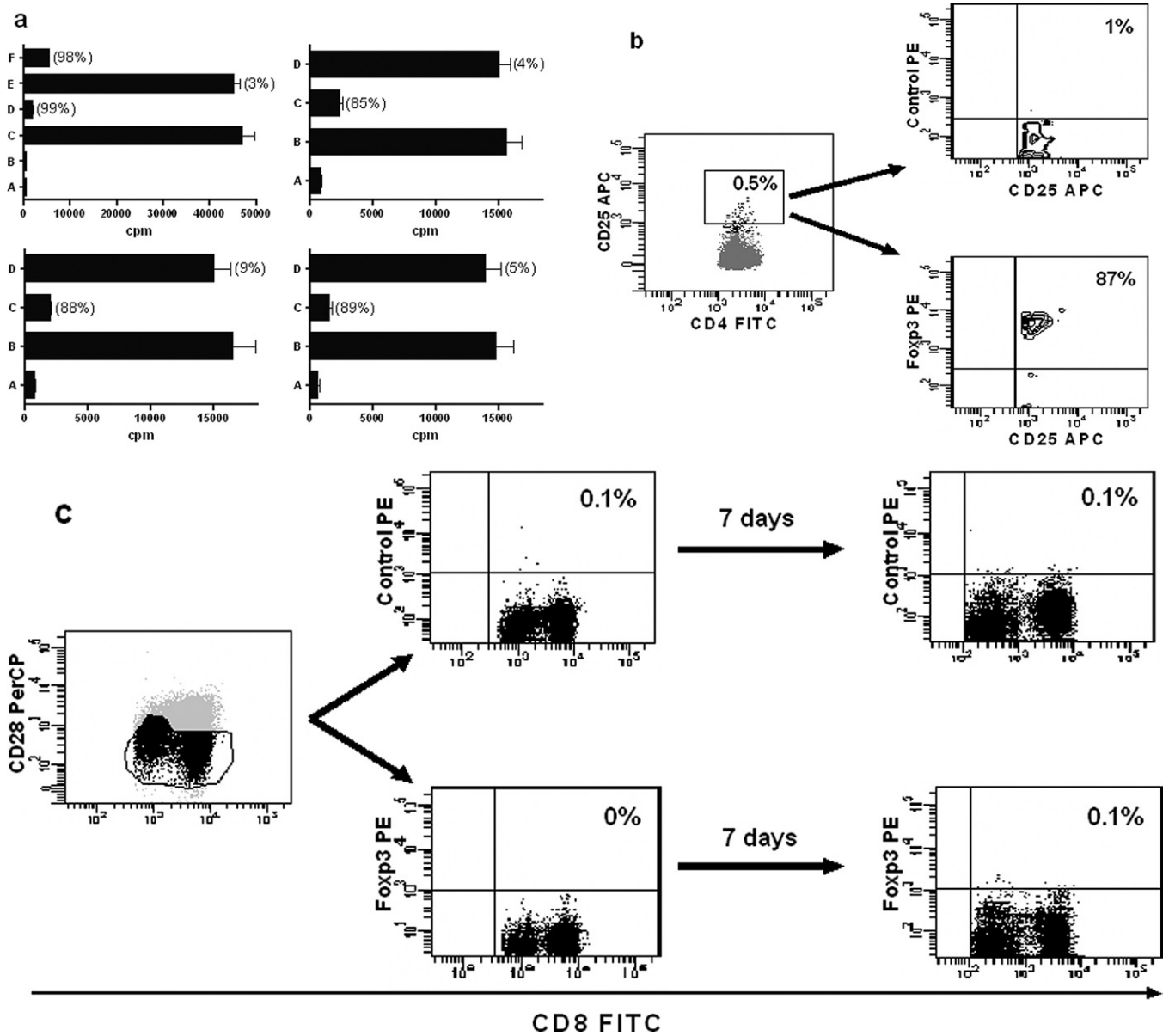

Fig. 1. Analysis of Foxp3 expression by CD8+CD28− and CD4+CD25+ Treg. (Panel a) CD8+CD28− Treg inhibit anti-CD3 mAb induced PBMC proliferation as well as that of antigen-specific T-cell lines. (Top left graph) (A) irradiated PBMC (1×10^5 cells/well); (B) irradiated PBMC plus nonirradiated autologous PBMC (1×10^5 cells/well); (C) as in B plus anti-CD3 mAb; (D) as in C plus CD8+CD28− Treg (8×10^4 cells/well); (E) as in D plus an anti-IL-10 mAb (10 μg/ml); (F) as in C plus autologous CD4+CD25+ Treg (8×10^4 cells/well). The percentage of suppression of proliferative activity is indicated between parentheses in columns D and E. (Top right graph) (A) tetanus toxoid–specific CD4+ T-cell line (1×10^5 cells/well) plus autologous irradiated PBMC (1×10^5 cells/well); (B) tetanus toxoid–specific CD4+ T-cell line (1×10^5 cells/well) plus tetanus toxoid–pulsed (5 μg/ml of antigen in PBS for 1 hour at 37°C) autologous irradiated PBMC (1×10^5 cells/well); (C) as in B plus autologous CD8+CD28− Treg (8×10^4 cells/well); (D) as in C plus an anti-IL-10 mAb (10 μg/ml). The percentage of suppression of proliferative activity is indicated between parentheses on columns C and D. Bottom left graph: (A) Candida-specific CD4+ T-cell line (1×10^5 cells/well) plus autologous irradiated PBMC (1×10^5 cells/well); (B) candida-specific CD4+ T-cell line (1×10^5 cells/well) plus tetanus toxoid pulsed (5 μg/ml of antigen in PBS for 1 hour at 37°C) autologous irradiated PBMC (1×10^5 cells/well); (C) as in B plus autologous CD8+CD28− Treg (8×10^4 cells/well); (D) as in C plus an anti-IL-10 mAb (10 μg/ml). The percentage of suppression of proliferative activity is indicated between parentheses in columns C and D. (Bottom right graph) (A) PPD-specific CD4+ T-cell line (1×10^5 cells/well) plus autologous irradiated PBMC (1×10^5 cells/well); (B) PPD-specific CD4+ T-cell line (1×10^5 cells/well) plus tetanus toxoid pulsed (5 μg/ml of antigen in PBS for 1 hour at 37°C) autologous irradiated PBMC (1×10^5 cells/well); (C) as in B plus autologous CD8+CD28− Treg (8×10^4 cells/well); (D) as in C plus an anti–IL-10 mAb (10 μg/ml). The percentage of suppression of proliferative activity is indicated between parentheses in columns C and D. (Panel b) CD4+CD25+ Treg express Foxp3: CD4+CD25+ T cells purified from the peripheral blood of a healthy donor were gated and subsequently analyzed by an APC-conjugated anti-Foxp3 mAb or by its relative isotypic control mAb. The percentage of positive cells is indicated. (Panel c) CD8+CD28− Treg do not express Foxp3: CD8+CD28− T cells purified from the peripheral blood of a healthy donor were gated (left graph) and subsequently analyzed by an APC-conjugated anti-Foxp3 mAb or by its relative isotypic control mAb (middle graphs). The analysis was also performed on CD8+CD28− Treg generated after 7 days culture of CD8+CD28− T cells in the presence of IL-2 and IL-10 (right graphs). Data shown are representative of four experiments performed with cells from eight different individuals. Abbreviations as in text.