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Technical note

A novel method for detecting antigen-specific human regulatory T cells

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ABSTRACT

Antigenic epitopes recognized by FoxP3$^+$ regulatory T cells (Treg) are poorly defined, largely due to a lack of assays for determining Treg specificity. We have developed a novel approach for detecting human Treg specific to peptide antigen, utilizing down-regulation of surface CD3 as a read-out of antigen recognition. Culture conditions and re-stimulation time have been optimized, allowing the detection of even very rare Treg, such as those specific to tumor antigens.

1. Introduction

FoxP3$^+$ Treg play a critical role in negative regulation of the immune system (Wing and Sakaguchi, 2010), yet relatively little is known about their antigen specificity, and very few Treg epitopes have been precisely defined. This is partly due to a lack of assays suitable for determining the antigen specificity of Treg. The antigen specificity of effector T cells (Teff) is generally defined by stimulating the cells *in vitro* with peptide antigen and assaying for a function typical of that Teff population; generally, production of effector cytokines such as IFN-γ. Human Treg, on the other hand, fail to consistently produce any cytokines following antigen stimulation (Levings et al., 2002; Godfrey et al., 2005), the only possible exception being TGF-β which is difficult to detect, particularly using flow cytometry. The major function ascribed to Treg is suppression of proliferation of other T cells. However, measuring this activity is cumbersome and not suitable for the large-scale screening assays required for epitope identification. It is also uncertain how well this activity is linked to activation through the T cell receptor (TCR) (Szymczak-Workman et al., 2009). Clearly, novel approaches are required to identify and screen for Treg specific for defined antigens and antigenic epitopes.

In accordance with this need, we have developed a novel approach for detecting antigen-specific human Treg. This approach is based on the principle that T cells down-regulate the number of CD3/TCR complexes on their surface after interacting with specific antigen/MHC complexes (Valitutti et al., 1995). This response can be detected as a reduced level of cell surface CD3 staining by flow cytometry. Additional staining for the Treg markers CD25 and FoxP3 allows the Treg to be selectively gated during flow cytometric analysis, and the CD3 down-regulation response within this population specifically examined. By combining this approach with a prior culture step, even Treg of very low frequency, such as those specific to tumor antigens, can be detected.

2. Methods

2.1. Patient samples

Patient PBMC samples used in this study were obtained as part of a clinical trial involving patients with histologically confirmed stage IV (metastatic) or unresectable stage III malignant melanoma. Patients received a vaccine comprising full-length recombinant NY-ESO-1 protein formulated with

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ISCOMATRIX® adjuvant. Details of the patients, vaccine and treatment course are described in detail elsewhere (Nicholaou et al., 2009).

2.2. Antibodies and flow cytometry

Antibodies specific to CD3, CD4 and CD25 were obtained from BD Biosciences (Franklin Lakes, NJ) and antibody to FoxP3 (clone 236A/E7) was obtained from eBioscience (San Diego, CA) as part of a kit containing fixation and permeabilization reagents, which were used according to the manufacturer's recommendations. Flow cytometry was performed using a BD FACSCanto II instrument, and data was analyzed using FlowJo software (TreeStar Inc., Ashland, OR). 'Live/Dead' vital dye (Invitrogen, Carlsbad, CA) was used according to the manufacturer's recommendations to gate out dead cells from all analyses.

2.3. Culture medium, cytokines and peptides

Culture medium used throughout was RPMI-1640 supplemented with 2 mM Glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 100 μM non-essential amino acids and 50 μM 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA). Human serum from the Australian Red Cross was pooled from several healthy donors, while fetal calf serum (FCS) was obtained from Invitrogen; both were added to the base culture medium to 10% to generate complete medium. Serum was not heat-inactivated, in order to preserve the activity of serum proteases required for trimming peptides prior to loading onto MHC (Kozlowski et al., 1993). Note that human serum was used for all cultures unless specifically indicated. IL-2 ('Proleukin') was obtained from Prometheus (San Diego, CA). NY-ESO-1 peptides were synthesized by the Department of Chemistry, Auckland University (Auckland, New Zealand) and Chiron Mimotopes (Clayton, VIC, Australia). For culture, a library of 28 peptides, each 18aa in length, was used, collectively covering the entire sequence of the NY-ESO-1 protein. The same peptides were used individually for re-stimulation.

2.4. Detection of antigen-specific Treg using CD3 down-regulation

PBMC were isolated from blood by Ficoll-Paque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and cryopreserved in 10% dimethyl sulfoxide (DMSO) until required. Cryopreserved patient PBMC were rapidly thawed and diluted with an excess of complete medium containing 50 U/ml DNase (Sigma) to assist with breaking up cell clumps. After 5 min' incubation at room temperature, cells were washed and re-suspended to a concentration of $2.5–3.5\times10^7$ cells/ml. Aliquots of 200 μl were dispensed into a 24-well plate and incubated with the library of NY-ESO-1 18aa peptides (each peptide at 10^{-5} M final concentration) for 1 h at 37 °C. Cultures were then diluted ~10-fold with complete medium containing IL-2 (300 U/ml, unless otherwise indicated) and incubated at 37 °C for 14–28 days, as indicated. During this time, cultures were fed every 2–3 days using complete medium supplemented with IL-2 and split as required, taking care to maintain a reasonably high cell density at all times (~$1.5–2.5\times10^6$ cells/ml). To detect antigen-specific Treg following the culture period, a maximum of 1×10^6 cells were incubated at 37 °C in 100 μl of fresh medium containing peptide at 1×10^{-4} M for 16 h, unless otherwise indicated. Cells were then stained for CD3, CD4, CD25 and FoxP3 and analyzed by flow cytometry, gating on viable $CD4^+$ T cells.

3. Results

3.1. Optimal culture conditions for expansion of antigen-specific Treg

Fig. 1A shows an example of the method, in which peripheral blood mononuclear cells (PBMC) from a patient with malignant melanoma were examined for responsiveness to a short, 18 amino acid (18aa) synthetic peptide based on the sequence of the NY-ESO-1 human tumor antigen. The patient's PBMC were cultured with a pool of NY-ESO-1 18aa peptides for 21 days, to allow expansion of all NY-ESO-1-specific T cells to detectable numbers. This culture step was necessary because tumor antigen-specific T cells are generally too rare to be detected directly *ex vivo* (Jackson et al., 2004). The cultures were then re-stimulated with each NY-ESO-1 peptide individually and analyzed by flow cytometry. The example shown in Fig. 1A illustrates the response to re-stimulation with NY-ESO-$1_{79–96}$ peptide, which was one of five peptides which induced a response for this particular patient. After gating on $CD4^+$ T cells, a sub-population of $CD25^+FoxP3^+$ Treg could be clearly identified, enabling these cells to be gated and examined separately from the $CD4^+$ Teff ($FoxP3^-$) population. In the absence of peptide re-stimulation, expression of CD3 on both the Treg and Teff populations was uniformly high. In contrast, re-stimulation with NY-ESO-$1_{79–96}$ peptide generated a distinct subset of CD3-low (antigen-responsive) cells within the Treg population but not the Teff population. Further analyses have shown that the $CD4^+$ $CD25^+$ $FoxP3^+$ T cells studied here after 21 days culture have the phenotype and function of Treg, including reduced CD127 expression, induction of the TGF-β latency-associated peptide (LAP) upon activation and suppression of T cell proliferation (Ebert et al., manuscript submitted).

Treg have distinct growth requirements during *in vitro* culture compared to Teff. For example, they are strictly dependent on high doses of exogenous IL-2 (Hoffmann et al., 2004). We therefore sought to determine the optimal conditions for antigen-induced expansion of human Treg. For all of these studies, PBMC were obtained from a number of different patients with malignant melanoma and were pre-screened with the full panel of 28 NY-ESO-1 peptides to determine which peptides induced responses for each patient. The peptide or peptides which induced the largest response were then used for subsequent experiments. Each figure is representative of results from 2 to 3 individual patients tested in independent experiments.

Fig. 1B illustrates the effect of IL-2 concentration and serum type on the percentage of Treg specific to the NY-ESO-$1_{157–170}$ peptide. Strikingly, culture in the presence of 10% fetal calf serum (FCS) failed to allow detectable expansion of NY-ESO-1-specific Treg, regardless of the amount of IL-2 added. In contrast, culture in the presence of 10% pooled human serum and 75 U/ml IL-2 generated a clear population of NY-ESO-1-specific Treg. Increasing the concentration of IL-2 to 150 U/ml or