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Technical note

Blockade of LFA-1 augments in vitro differentiation of antigen-induced Foxp3$^+$ Treg cells

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ABSTRACT

Adoptive transfer of antigen-specific, in vitro-induced Foxp3$^+$ Treg (iTreg) cells protects against autoimmune disease. To generate antigen-specific iTreg cells at high purity, however, remains a challenge. Whereas polyclonal T cell stimulation with anti-CD3 and anti-CD28 antibody yields Foxp3$^+$ iTreg cells at a purity of 90–95%, antigen-induced iTreg cells typically do not exceed a purity of 65–75%, even in a TCR-transgenic model. In a similar vein to thymic Treg cell selection, iTreg cell differentiation is influenced not only by antigen recognition and the availability of TGF-β but also by co-factors including costimulation and adhesion molecules. In this study, we demonstrate that blockade of the T cell integrin Leukocyte Function-associated Antigen-1 (LFA-1) during antigen-mediated iTreg cell differentiation augments Foxp3 induction, leading to approximately 90% purity of Foxp3$^+$ iTreg cells. This increased efficacy not only boosts the yield of Foxp3$^+$ iTreg cells, it also reduces contamination with activated effector T cells, thus improving the safety of adoptive transfer immunotherapy.
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1. Introduction

In addition to thymic regulation, peripheral induction of a regulatory phenotype in conventional T (Tconv) cells provides protection from undesirable immune responses to self antigens. Adoptive transfer of in-vitro induced Foxp3$^+$ T regulatory (iTreg) cells forms an attractive approach to therapeutically restore the balance when healthy immunity is disturbed, i.e. in autoimmune disease. iTreg cells can be generated at very high purity by polyclonal stimulation of naive CD4$^+$CD62L$^+$ T cells with anti-CD3 and anti-CD28 antibody in the presence of TGF-β and IL-2 (Thornton et al., 2010; Verhagen et al., 2013a). Studies using iTreg cells obtained in this way from TCR-transgenic mice have demonstrated that antigen-specificity is an important factor in the functionality of transferred iTreg cells (DiPaolo et al., 2007; Chattopadhyay and Shevach, 2013) (personal observation JV, unpublished). Although iTreg cells have been the subject of intense investigation, the in vitro differentiation of antigen-specific iTreg cells, using cognate ligand rather than anti-CD3 and anti-CD28, at high purity remains a challenge. We have demonstrated previously that, in the Tg4 mouse model, iTreg cells can be generated using cognate Myelin Basic Protein (MBP) peptide as a stimulus but the level of conversion lags behind that achieved using antibody stimulation (65–75% vs 90–95%) (Verhagen et al., 2013a). This low purity not only limits the yield of Foxp3$^+$ iTreg cells, the contamination with activated Foxp3$^-$ T cells that may exert pro-inflammatory effector functions poses a potential risk when used for Treg cell-based immunotherapy.

Development of Foxp3$^+$ iTreg cells depends not only on TCR signals and the availability of TGF-β, but also on additional co-factors. For example, CTLA-4 has previously been suggested to be important for TGF-β-dependent Foxp3 induction (Zheng et al., 2006), although this finding was recently contradicted (Chattopadhyay and Shevach, 2013). In

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this study, we compared the effects of ligation or blockade of a number of costimulatory and adhesion molecules involved in T cell activation and regulation on Foxp3 induction in vitro. Interestingly, we found that blockade of Leukocyte Function-associated Antigen-1 (LFA-1) with monoclonal antibody augmented antigen-induced Foxp3 expression, giving rise to iTreg cells approximately 90% Foxp3$^+$. LFA-1 is an integrin that, through interaction with its ligand ICAM-1, mediates T cell-APC interaction and is involved in stable formation of the immunological synapse (Shimizu, 2003). It, therefore, controls the avidity of the activation signals received through the T cell receptor and costimulatory molecules. Although signaling through LFA-1 itself is less well defined, LFA-1 ligation has been shown to make CD4$^+$ T cells refractory to TGF-β signaling through upregulation of Smad7, SKI and SMURF2 (Verma et al., 2012). We have not been able to establish if the effect of anti-LFA-1 during iTreg differentiation follows a direct or indirect impact of LFA-1 on Foxp3 induction but the result is in line with previous findings; the prevention of allogeneic transplant rejection by treatment with anti-LFA-1 has been shown to be associated with an increased frequency of CD4$^+$Foxp3$^+$ Treg cells in the graft-draining lymph nodes (Reisman et al., 2011). Here, we demonstrate that our method induces antigen-specific iTreg cells of high purity that successfully protect against CNS autoimmune disease.

2. Materials and methods

2.1. Mice

B10.PL, Tg4, Tg4 CD45.1$^+$ and Tg4 Foxp3^{gfp} (Verhagen et al., 2013a) mice were bred and kept under specific pathogen-free conditions. All experiments were carried out under a UK Home Office Project Licence and were subject to assessment by the University of Bristol ethical review committee.

2.2. Peptide

The acetylated N-terminal peptide of murine MBP, Ac1-9 (Ac-ASQKRPSQR) and its high MHC affinity variant (Ac-ASQYRPSQR) were custom synthesized (purity > 85%; GL Biochem (Shanghai) Ltd.)

2.3. Naive T cell isolation

CD4$^+$CD62L$^+$ naive T cells were isolated magnetically from splenocytes using a naive T cell isolation kit (Stemcell Technologies) according to the manufacturer's recommendations.

2.4. iTreg cell differentiation

CD4$^+$CD62L$^+$ naive splenic T cells were cultured in vitro for 7 days in RPMI medium supplemented with 5% FCS, in the presence of 100 U/ml rhIL-2 (R&D systems) and 10 ng/ml rhTGF-β_1 (Peprotech). Cells were stimulated either with anti-CD3e (1 μg/ml) and anti-CD28 (2 μg/ml) plate-bound antibody (both from eBioscience) or MBP Ac1-9 peptide in the presence of irradiated B10.PL splenocytes used as antigen-presenting cells. Where indicated, functional grade antibody to LFA-1 (M17/4, Biolegend or eBioscience), CTLA-4 (9H10, eBioscience), PD-1 (J43, BioXCell), LAG3 (C9B7W, BioXCell) or IL-10R (1B1.3A, BioXCell) was added either plate-bound or soluble in the medium at 10 μg/ml for the duration of the culture. The level of FoxP3 induction was assessed by flow cytometry.

2.5. Flow cytometry

Flow cytometric analysis was performed using an LSR II or Fortessa X20 flow cytometer (BD). Cell phenotypes were analyzed using combinations of anti-FoxP3-PE, —efluor450 or –APC, anti-CD45.2-PerCPCy5.5, anti-CD45.1 PE-Cy7, anti-CD62L-PE-Cy7, anti-Ki67-ef450, anti-CD4-AlexaFluor700 (all from eBioscience), anti-Neuropilin-1-PE or —APC, anti-LFA-1 (clone 2D7)-PE, anti-Helios-FITC, and anti-CD103-PerCPCy5.5 (all from Biolegend) antibodies. Fixable viability dye eFluor780 (eBioscience) was used in all experiments to exclude dead cells. Cell proliferation dye-ef450 (CPD-ef450, eBioscience) was used to visualize cell divisions or calculate division and proliferation indexes. Results were analyzed using FlowJo analysis software (Tree Star, Inc.).

2.6. Demethylation analysis

Demethylation analysis of the foxp3 CNS2 region was carried out by EpigenDX, assay ADS568. The heat map matrix was created using the free online tool on http://www.chibi.ubc.ca/matrix2png/bin/matrix2png.cgi. Each block in the heat map represents the mean of 3 individual samples per condition. Female mice were used for the analysis. Therefore, the level of methylation is relative, with the highest level of methylation in any CpG in the CD4$^+$ Tconv cell group set as the maximum and the lowest level in any CpG in the CD4$^+$CD25$^+$ Treg cell group as the minimum.

2.7. Induction and scoring of EAE

Tg4 mice, with 5×10^6 iTreg cells in PBS or PBS only transferred i.p. on day —3, were primed subcutaneously at the base of the tail with 200 μg of MBP Ac1-9 in 0.1 ml of a sonicated emulsion consisting of an equal volume of complete Freund's adjuvant (CFA) and PBS, containing 4 mg/ml of heat-killed *Mycobacterium Tuberculosis* (both from Difco). On days 0 and 2, 200 ng of Pertussis toxin (Sigma Aldrich) was administered intraperitoneally in 0.5 ml of PBS. EAE was assessed twice daily with the following scoring system: 0, no signs; 1, flaccid tail; 2; impaired righting reflex and/or gait; 3, hind limb paralysis; 4, forelimb and hind limb paralysis; 5, moribund.

2.8. Statistical analysis

Data were analyzed for statistical significance using GraphPad Prism software.

3. Results and discussion

3.1. Foxp3 expression can be induced with peptide even in low-frequency antigen-specific Tconv cells

In experimental settings, antigen-specific iTreg cells are commonly generated from murine TCR-transgenic CD4$^+$ T cells

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