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Research paper

Large scale preparation of human MHC class II$^+$ integrin β_1^+ Tregs

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ABSTRACT

The human CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cell population (Tregs) contains both MHC class II$^+$ and MHC class II$^-$ cells. MHC class II$^+$ Tregs belong to the integrin $\alpha_4\beta_1^+$ subpopulation and exclusively execute contact-dependent suppressive activity. Here we present a method optimized for isolation of these MHC class II expressing Tregs from large leukaphereses products using magnetic microbeads that achieves a reproducible purity of more than 90% and enables the use of this small-sized Treg population in pre-clinical application and basic research.

1. Introduction

Human thymus-derived CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs) contain several phenotypically different subpopulations (Stassen et al., 2004; Valmori et al., 2005; Fritzsching et al., 2006; Baecher-Allan et al., 2006; Borsellino et al., 2007; Battaglia et al., 2008; Nagata et al., 2009) which exert different effector functions (Stassen et al., 2004; Tang and Bluestone, 2008) and supposedly represent distinct differentiation states (Miyara et al., 2009). Because Treg subpopulations have been identified separately, only very little is known about their interdependency. Moreover, because of their limited availability, the therapeutic potential of individual human Treg subpopulations is largely unknown (Ermann et al., 2005).

MHC class II expression defines a population of typically 20–30% of all Tregs (Baecher-Allan et al., 2006). Although the functional role of MHC class II expression on these cells is unclear, it was found to strictly correlate with contact-dependent suppressive activity and possibly marks a state of activation. Because of their more rapid suppressive activity compared to the overall Treg population, MHC class II expressing Tregs seem to be particularly well suited for therapeutic approaches. However, the therapeutic value of these cells has not been studied, presumably because of the difficulty to isolate them in sufficient quantity. We here present an isolation procedure optimized for high quantity and high purity isolation of MHC class II$^+$ Tregs from large scale leukaphereses products that enables their use in pre-clinical application and basic research. Furthermore, we demonstrate that MHC class II$^+$ Tregs almost exclusively express integrin $\alpha_4\beta_1$ and thus overlap with the previously described subpopulation of $\alpha_4\beta_1^+$ human Tregs whereas MHC

Abbreviations: Tregs, human CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells.

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II$^-$Tregs are heterogeneous and contain several populations including the $\alpha_4\beta_7^+$ Tregs (Stassen et al., 2004).

2. Material and methods

2.1. Leukaphereses samples

Leukaphereses were obtained from adult healthy volunteers following approval by the local ethical committee (Landesärztekammer Rheinlandpfalz No 837.029.05 (4687)).

2.2. Isolation of MHC class II$^+$ Tregs

PBMC were isolated from Leukaphereses (up to 1.5×10^{10} whole cells) by density gradient centrifugation. CD4$^+$ T cells were isolated from PBMC by incubating the cells with a limited concentration of CD4 multisort beads (4 μl/10^7 cells, Miltenyi Biotec, Bergisch-Gladbach, Germany) and magnetically separating the cells on 6–7 MS columns (Miltenyi) on midi MACS separators (Miltenyi) in parallel. Resulting CD4$^+$ T cells were of high purity (>95%, average yield 25% of PBMC). In a second step, CD25^{high} cells were magnetically separated from previously isolated CD4$^+$ T cells by positive selection with a restricted amount of CD25-microbeads (1 μl/10^7 CD4$^+$ T cells, Miltenyi Biotec). CD25^{high} cell purity was typically ≥97%.

Remaining CD4$^+$CD25$^-$ cells from the second isolation step still contained few contaminating CD25$^+$ cells. To obtain CD4$^+$CD25$^-$ cells with high purity, contaminating CD25$^+$ cells were depleted with CD25-Dynabeads (0.5 beads per cell, final purity: greater than 98%).

For purification of MHC class II$^+$ Tregs, isolated CD4$^+$CD25^{high} cells were incubated with Dynabeads coupled with the mAb MZ05 directed against HLA-DP (see supplemental figure 1) at a ratio of 1 bead per cell and separated on a magnetic particle concentrator (Invitrogen). To increase efficiency, separation was repeated once on the MHC class II$^-$ cell fraction.

2.3. Suppression assay

CD4$^+$CD25$^-$ Th cells were plated at 1×10^5/well together with 3×10^5/well irradiated (50 Gy) PBMC in presence of 0.5 μg/ml anti-CD3 mAb (clone OKT-3). Sorted Treg populations were added to the cultures at different ratios starting at 1×10^5/well (Treg: Th cells 1:1). Alternatively, the suppressive activity of isolated Tregs was measured in a feeder-cell free suppressor assay using 5×10^4 T cells/well stimulated with plate bound anti-CD3 mAb (1 μg) and 2 μg anti-CD28 mAb respectively. To determine proliferation, [^{3}H] thymidine was added on day three for a 16 h-pulse. All assays exhibited less than 10% SE and were repeated at least three times with T cell subsets from different donors. Differences in proliferation between samples were analyzed by Student's *t*-test. A *P*-value of ≤0.05 was considered significant.

2.4. Flow sorting

Integrin $\alpha_4\beta_1^+$ and $\alpha_4\beta_7^+$ Tregs were sorted from magnetically isolated MHC class II$^+$ and MHC class II$^+$ Treg subsets (2.2) by three-color sorting (FACS Aria, BD Biosciences). Cells were stained with fluorescence-labeled anti-Integrin β_7, anti-Integrin β_1^+ and anti-MHC class II (clone MZ05) mAb. Cells were gated on small lymphocytes by forward and side scatter, then on MHC class II and integrin expression. Sorted cells were typically >98% pure in post-sort flow cytometry analysis.

2.5. MHC class II staining and preparation of MHC class II-specific Dynbeads

For staining of MHC class II and generation of MHC class II-specific magnetic beads, an antibody generated by immunizing mice with human Tregs (Becker et al., 2010) designated MZ05 was used. Immunoprecipitation followed by MS/MS mass spectometry identified the HLA-DP β-chain as the respective antigen of MZ05. Epitope mapping by a gridded array of synthetic peptides consisting of 12-residue peptides

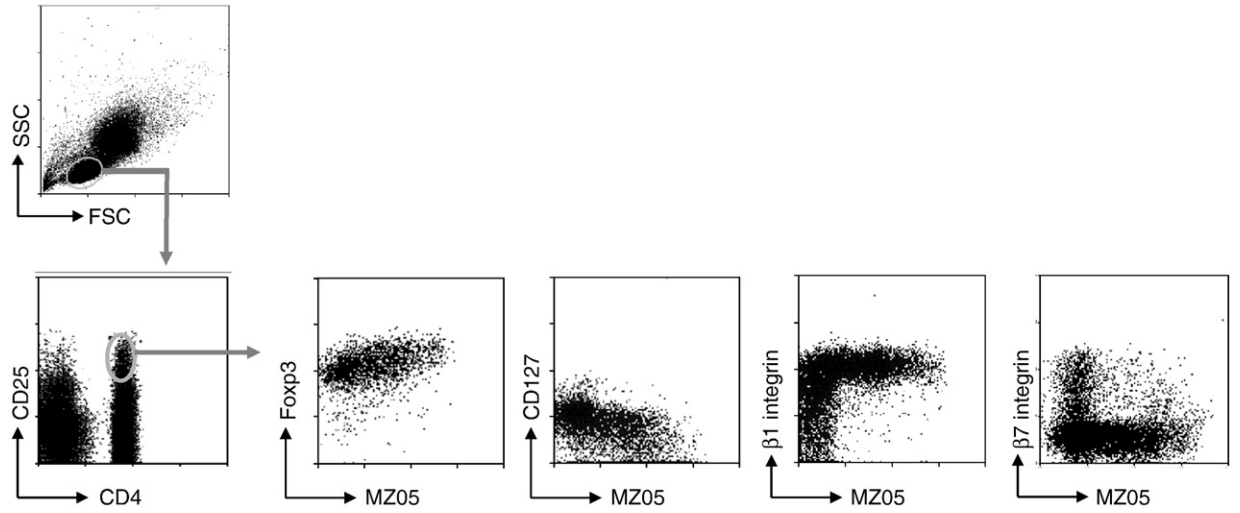

Fig. 1. Phenotype of MHC class II expressing Tregs in human PBMC. Flow cytometric analysis of the Treg associated markers Foxp3, CD127, β_1 and β_7 integrins in CD4$^+$CD25^{high}MHC II$^+$ (MZ05$^+$) T cells in human blood. Data are representative of 10 donors.