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Induced regulatory T-cells (iTregs) generated by activation with anti-CD3/CD28 antibodies differ from those generated by the physiological-like activation with antigen/APC

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ABSTRACT

Regulatory T-cells (Tregs) are responsible for homeostasis of the immune system, as well as for inhibition of pathogenic autoimmune processes. Induced-(i)-Tregs, can be generated in vitro by activation of CD4 cells in the presence of TGF-β. A commonly used activation mechanism is by antibodies against CD3 and CD28. The physiological-like activation of T-cells, however, is with the specific target antigen presented by antigen-presenting cells (APC). The two modes of activation have been considered to yield the same populations of iTregs. Here, we compared between iTreg populations generated by either one of the two methods and found differences between their capacities to inhibit T-lymphocyte proliferative response, their expression of cell surface antigens and particularly, in their transcript expression profiles of certain chemokines and chemokine receptors. Our data thus indicate that iTregs generated by activation with anti-CD3/CD28 antibodies cannot be considered identical to iTregs generated by antigen/APC.
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1. Introduction

The pivotal role of regulatory T cells (Tregs) in maintenance of homeostasis of the immune system is well established [1,2]. Treg cells are a subset of CD4 lymphocytes that express the specific transcription factor fork head box P3 (Foxp3) and are readily identified by this feature [1–4]. Additional studies have identified two sub-populations of Tregs, namely, thymic derived "natural" (nTregs) and peripherally generated "induced" (iTregs), also known as "adaptive Tregs" [1–6]. In addition to their origin, the two subpopulations differ in their surface phenotype, costimulation and cytokine dependence, T cell receptor (TCR) repertoire and homing behavior [2,7,8]. It is also proposed that development of iTreg cells may be driven by the need to control immune response against environmental challenges, whereas nTregs are involved in maintenance of self-tolerance and prevention of autoimmunity [7].

In vitro investigations have defined the culture components that transform naïve CD4 cells and induce them to acquire the features of iTregs, i.e., expression of Foxp3 and immunosuppressive capacity. The essential cytokine for the transformation is TGF-β and the process is enhanced by IL-2 [5,9–11]. In addition, the induction of iTreg features requires activation of the T-cells and a commonly used activation mechanism has been antibodies against CD3, or a combination of anti-CD3 and anti-CD28 antibodies [5,10–12]. The physiological activation mechanism, however, is by the specific antigen, presented by antigen presenting cells (APC). The number of antigen-specific iTreg cells is minute in wild type mice and, therefore, their features could only be examined by using TCR-transgenic (Tg) mice, in which the majority of T-cells express the same receptor. The availability of TCR Tg mice made it possible for us to compare in previous studies between polarized lineages

Abbreviations: Treg, regulatory T cells; Foxp3, fork head box P3; nTregs, natural Tregs; iTregs, induced Tregs; TCR, T cell receptor; Tg, transgenic; APC, antigen presenting cell; Th, T-helper; HEL, hen egg lysozyme; CD, cluster of differentiation; HA, activated by HEL and APC; PbAb, activated by plate-bound anti-CD3/CD28 antibodies; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; LFA-1, lymphocyte function-associated antigen 1; PSGL-1, P-selectin glycoprotein ligand 1.

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of T-helper (Th) cells, Th1 and Th17, generated in vitro by either one of the two modes of activation [13,14] and we used here this approach to compare between iTregs generated by the two approaches.

In the experimental system we use, naïve TCR Tg T-cells specific against hen egg lysozyme (HEL) are polarized in vitro toward the required phenotype by their activation in the presence of the phenotype-specific cytokine cocktails [13,14]. For the activation process, we employ either one of two mechanisms, i.e., HEL presented by APC ("HA"), or plate-bound anti CD3/CD28 antibodies ("PbAb") [15]. In the present study we generated in vitro lineages of iTregs by activation during polarization with either the HA or the PbAb mechanisms and compared these lineages for several biological features. Considerable differences were observed between the two types of iTregs for most tested parameters. Of particular interest were the findings that (i) the population of iTregs cells generated by HA had lower proportions of cells expressing FoxP3 than the PbAb population, but exhibited higher immunosuppressive capacity; (ii) moderate differences were observed between the two populations in their expression of surface markers, but (iii) profound differences were seen in their expression profiles of selected chemokines and chemokine receptor transcripts.

2. Materials and methods

2.1. Mice

The mice used in this study were (FVB/N × B10.BR) F1 hybrids, transgenically expressing HEL-specific TCR by their T cells ("3A9" mice), or non-transgenic wild type syngeneic animals. See Ref. [13] for more details. The mice were housed in a pathogen-free facility and all manipulations were performed in compliance with the NIH Resolution on the Use of Animals in Research.

2.2. Cytokines, antibodies and antigen

IL-6, TGF-β, PE-conjugated anti-CCR6, anti-CXCR3, anti-CXCR6, anti-CCR2 antibodies and their corresponding isotype controls were provided by R&D Systems (Minneapolis, MN). IL-1α was from PeproTech (Rocky Hill, NJ); anti-IFN-γ (clone R4-6A2) was from Harlan Bioproducts for Science (Madison, WI); anti-IL-4 (clone 11B11) and recombinant human IL-2 (rIL-2) were from NCI-Frederick Repository; IL-12 and HEL were purchased from Sigma–Aldrich (St. Louis, MO). Anti-CD3, anti-CD28 and anti-IL-12 antibodies; IL-4; FITC-conjugated anti-CD4, PE-conjugated anti-CD4, anti-α4β7, anti-CD103 (α E β 7), anti-CD62L, anti-CXCR4, anti-LFA-1, anti-PSGL-1 antibodies and corresponding isotype controls were purchased from BD Bioscience (San Jose, CA). PE-conjugated anti-CCR9, anti-CCR7, anti-CCR5 antibodies, APC-conjugated anti-Foxp3 antibodies and corresponding isotype controls were from eBioscience (San Diego, CA). A clonotypic mAb specific for the TCR of 3A9 mice, designated "1G12", a gift from E. Unanue (Washington University, St. Louis, MO), was conjugated with FITC. PE-conjugated anti-CCR4 antibody and its isotype control were from BioLegend (San Diego, CA).

2.3. Generation of iTreg subpopulations

T cells from spleen and lymph node cells of naïve 3A9 mice were enriched by using T cell purifying columns (R&D Systems) and were further purified by MACS microbeads (Miltenyi Biotec, Auburn, CA), to obtain naive CD4+ T cells. Antigen (HEL)-specific iTreg cells were generated as follows: CD4+ T-cells from 3A9 mice (1G12+) were cultured in 12-well plates (Corning Glass, Corning, NY) at 5×10^5/ml cells per well in a volume of 2 ml RPMI 1640 medium supplemented with 10% fetal calf serum, antibiotics and 50 μM 2-ME ("complete medium"). The cultures were polarized by TGF-β (5 ng/ml), along with IL-2 (50 IU/ml) and activated by either HEL (2.0 μg/ml) and 25×10^5/ml APCs (irradiated [30 Gy] syngeneic wild-type naive splenocytes) ("HA-iTregs"), or by plate-bound anti-CD3/CD28 antibodies (1 μg/ml for both antibodies, incubated for 1 h before removal of the supernatant) ("PbAb-iTregs"). In additional experiments, reported as Supplemental Data, different concentrations of HEL and of the coating anti-CD3/CD28 antibodies were used, as indicated. Following incubation for 3 days, the cells in each well were split into two wells with daily replacement of half of the medium (1 ml/well) with fresh complete medium containing 50 IU/ml IL-2. Percentage of Foxp3 + cells was 60–80% in cultures activated by HA and over 95% in cultures activated by PbAb (Fig. 1E, below). All of the iTreg cells used in this study were harvested on day 6, except for those used for flow cytometric analysis of surface antigens, harvested on day 7.

2.4. Protocols for polarization of Th lineages

The polarization conditions of naïve HEL-specific CD4+ T cells toward Th1, Th17 and Th9 cells were similar to those described previously [13,16]. In short, naive CD4+1G12+ T cells sorted by a FACS Aria II flow cytometer (BD Biosciences) from spleen and lymph nodes of 3A9 mice were cultured at the indicated concentration, in complete RPMI-1640 medium with 2 μg/ml HEL and APCs under conditions specific for Th1 (10 ng/ml IL-12 and 10 μg/ml anti-IL4), Th17 (3 ng/ml TGF-β, 10 ng/ml IL-6, 5 ng/ml IL-1α, 20 μg/ml anti-IFN-γ, 10 μg/ml anti-IL12 and 10 μg/ml anti-IL4), or Th9 (1 ng/ml TGF-β and 10 ng/ml IL-4).

2.5. Inhibition of Th proliferation by iTregs

The inhibitory effect of iTreg cells was determined according to their capacity to inhibit the proliferation of different Th lineages in culture during their polarization process, as follows: naïve 3A9 CD4 cells were cultured in Th1, Th17 or Th9 polarizing conditions, as described above in 96-well, round-bottom plates (U96 MicroWell Plates (NUNC, Denmark)), at 5×10^4 cells/well, in the presence of 2 μg/ml HEL and 15×10^4/well APCs. iTregs tested for their suppressive capacity were added to the cultures at increasing numbers, as indicated. Proliferation levels were determined on day 3 in culture, by ^{3}H-thymidine incorporation, with the pulse given for the last 8 h of culture.

2.6. Flow cytometric analysis: surface antigens and Foxp3 expression

HA- and PbAb-iTreg cells harvested on day 7 of culture, were collected for surface antigen staining and intracellular staining of Foxp3 according to the manufacturer's instructions (eBioscience). Flow cytometry was performed by a FACS Calibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Dead cells were excluded by gating on live cell population, using the FSC/SSC plotting.

2.7. Quantitative (q)-PCR analysis

Transcript levels of tested genes in iTreg populations were assessed by qPCR, as described elsewhere [14,16], using reagents and methods according to the manufacturer's instructions (Applied Biosystems).

2.8. Statistical analysis

Two tailed Student's *t* tests were used for determining the significance of data comparison.