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Identification of a human Th1-like IFNγ-secreting Treg subtype deriving from effector T cells

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ABSTRACT

Characteristics and function of effector T-cells with regulatory properties (induced Treg, "iTreg") in humans are ill defined. Here we report that a proportion of activated, initially CD4(+)CD25(−)CD127(+) effector T-cells from human peripheral blood can convert into T-cells with regulatory activity while concomitantly secreting IFNγ. Upon short-term culture *in vitro* these cells expressed a panel of common Treg markers, including FOXP3, CD25, GITR, HLA-DR and CTLA-4 in parallel with the Th1-specific transcription factor T-bet. Despite their own IFNγ secretion they effectively suppressed IFNγ secretion in effector T cells in parallel with inhibition of their proliferation. Highly purified IFNγ(+)iTreg shared many functional properties with nTreg: Their suppressive activity was antigen-independent, contact-mediated and cytokine-independent. Of note, in contrast to nTreg an inhibitor of TGF-β1 signalling promoted the proliferation of IFNγ(+)iTreg, without abrogating their suppressive function. In addition *in vivo* in tonsils of patients with chronic tonsillitis an IFNγ-secreting subpopulation of the CD4(+)CD25(−)CD127(+) CD45RA(−) memory T helper cell population was detected, which exhibited regulatory properties as well.

Our results support the existence of Th1-like adaptive Tregs in humans that express a robust regulatory phenotype, comparable to nTreg and at the same time share characteristics of Th1 cells. According to our *in vitro* data IFNγ(+)iTreg can emerge from activated effector T cells and downregulate Th1-mediated immune responses, supporting the hypothesis of effector T cell plasticity as a means for proper initiation and self regulation of inflammatory processes.

This report characterizes a new subpopulation of human adaptive regulatory T-cells that derive from effector Th-cells and concomitantly express Th1-specific T-bet and IFNγ with Foxp3.

1. Introduction

The immune homeostasis is controlled by mechanisms of central and peripheral tolerance. Central tolerance involves the deletion of self-reactive T-cells in the thymus at an early stage of development [1,2]. Peripheral tolerance -amongst other mechanisms- is maintained by a unique subset of naturally occurring CD4(+)CD25(+)T regulatory cells (nTreg) which suppress activation and effector functions of auto-reactive T-cells that have escaped other mechanisms of tolerance. This has been evidenced by adoptive transfer experiments in which these cells effectively prevented autoimmune diseases in mice [3]. Upon activation nTregs suppress proliferation and cytokine-production of conventional T-cells in an antigen non-specific, contact-dependent and cytokine-independent process that has not yet been completely defined [4,5]. The transcription factor foxp3 has been identified as master regulator gene involved in development and function of nTreg. Mutations in this gene cause functional impairment of nTregs and lead to the IPEX syndrome in humans [6,7]. Besides of FOXP3 high expression level of CD25 ($CD25^{high}$) together with low expression level of CD127 serve to identify nTreg in humans and distinguish them from activated T-cells [8–10].

In addition to nTreg adaptive or induced Treg (iTreg) have been found *in vivo* and *in vitro* which evolve independent of thymic control mechanisms during immune responses [11,12]. In general

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iTreg can be both FOXP3-positive or -negative and can be categorised into 2 major subsets based on their immunosuppressive mechanisms, involving the cytokines IL-10 (Tr1 cells) [13] or TGFβ (Th3 cells) [14]. This stands in contrast to nTreg, that mediate suppression primarily in a contact-dependent manner independent of cytokines.

The majority of information about iTreg subtypes has been generated from mouse experiments. Although studies in humans show that functionally suppressive iTreg can be reproducibly generated from activated CD4(+)CD25(−) T helper cells *in vitro* [15–17], data regarding human iTreg subtypes and their development and function *in vitro* and *in vivo* are still limited [18,19].

Here we report the development of a human IFNγ-secreting regulatory CD4(+)T-cell subset from CD4(+)CD25(−) effector T-cells upon optimal stimulation *in vitro*. These IFNγ-secreting CD4(+)Treg expressed both the nTreg-specific transcription factor Foxp3 as well as the Th1 specific transcription factor T-bet in a stable manner. In contrast to many other adaptive Treg-subtypes, their suppressive effect involved a cytokine-independent, contact-dependent mechanism, comparable to nTreg. Of note, IFNγ-secreting CD4(+)Treg could also be detected within the antigen-experienced fraction of human tonsillar Th-cells of patients suffering from chronic tonsillitis, suggesting that such Th1-like Treg not only exist *in vitro* but also appear *in vivo* during the course of an immune response, thereby complementing recent observations in mice [20].

2. Materials and methods

2.1. Reagents

Recombinant human IL-12, IL-4, IL-23, TGF-β, LAP was obtained from R&D systems, IL-2, IL-6 from Peprotech and IL-21 from Biosource International. The following antibodies were used for flow cytometry: FITC-anti-CD45RA, -CD45RO, -CD103, -CD62L, -CD95, -IFNγ, PE-anti-CD25, -CD127, -ICOSL, -CD28, -CCR4, -CCR7, -IL-4, -CD152, APC-anti-CD4, Biotin-anti-CD127 (BD Biosciences), FITC-anti-CD4, -CD25, -CD54, -HLA-DR, PeCy5-anti-CD25 (Beckmann coulter), FITC-anti-CD134, -CD161, PE-anti-ICOS, -PDL-1, -PD-1, -IL-4, -IL-17, -Foxp3 (PCH101) (eBioscience), Alexafluor 488-anti-Foxp3 (259D) (Biolegend), FITC-anti-CD137 and PE-anti-CCR5 (Serotec), FITC-anti-GITR (R&D systems), PE-anti-mTGF-β (IQ products), FITC-anti-CD18, -CD58, PE-anti-CD11a (Immunotools), APC-anti-CD45RA (MEM-56) (Invitrogen). Antibodies used for blocking and neutralization experiments: anti-human IL-4 (MP4-25D2), anti-IFNγ (B27), anti-IL-12 (C8.6) (BD Biosciences), anti-IL-2Rα (22722.2), anti-IL-2Rβ (27302), anti-IL-10 (23738), anti-TGF-ß1,2,3 (1D11), anti-polyclonal GITR (R&D systems), anti-B7-H1 (MIH1) (eBiosciences), anti-HLA-G (87G) (Exbio), anti-CD11a (MEM-25) (Immunotools) and CTLA4-Ig (Chimerigen Laboratories). Antibodies used for stimulation: anti-CD3 (OKT3) (American Type Culture Collection, ATCC) and anti-CD28 (CD28.2) (eBioscience). The inhibitor for NOs L-NAME (Nitro-Arginine Methyl Ester Hydrochloride), (Sigma) and TGF-β receptor type I kinase (ALK5) (Calbiochem) [21] were used in cultures.

2.2. Cell culture

T-cell isolation: PBMCs were isolated from heparinized peripheral blood by Lymphocyte-separation-media (LSM, PAA Labs) via density gradient centrifugation. CD4(+)T-cells were isolated from PBMCs with a negative CD4(+)T-cell isolation kit (Miltenyi Biotec). Enriched CD4(+)T-cells were stained with anti-CD4-APC or -PC5, anti-CD25-FITC and anti-CD127-PE and sorted with a FACS-VantageSE cell-sorter (BD Biosciences) for CD4(+)CD127(+) CD25− responder T-cells (Tresp) and CD4(+)$CD25^{high}CD127^{-/low}$ nTreg-cells (natural regulatory T-cell). Cells were grown in RPMI 1640 (PAA Labs) with 5×10^{-5} M 2-mercaptoethanol (2-ME)(Sigma), 1% nonessential amino acids, 2 mM glutamine, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 50 U/ml penicillin and 50 μg/ml streptomycin (all from PAA Labs). Since in preliminary experiments a higher expansion rate of iTreg was observed with cell culture media containing autologous serum versus FCS or no serum (Suppl.Fig.1A) most of our experiments were supplied with 10% autologous serum (with exception of MLR and tonsil experiments).

Generation of induced regulatory T-cells (iTreg): 2×10^5 Tresp sorted with a flow cytometry cell sorter were activated with anti-CD3mAb (1 μg/ml) and 1×10^6 irradiated (30Gy) autologous PBMC in a 24-well plate for 3 days, then fed with IL-2 (50 U/ml) every other day. As control, flow cytometric sorted autologous 2×10^5 nTreg were cultured with anti-CD3mAb (1 μg/ml), anti-CD28mAb (1 μg/ml), 2×10^6 irradiated (30 Gy) autologous PBMC and IL-2 (100 U/ml). Cells of both groups were split with fresh medium containing IL-2 every other day.

Generation of Th cell clones: 1×10^6 CD45RA(+)Tresp sorted with a flow cytometry cell sorter were cultured in a 24-well plate with coated anti-CD3 (OKT3) (3 μg/ml) and soluble anti-CD28 (3 μg/ml) for 3 days in the presence of IL-12 (25 ng/ml) and anti-IL-4 (10 μg/ml) for Th-1 or IL-4 (25 ng/ml), anti-IL-12 (10 μg/ml) and anti-IFNγ (10 μg/ml) for Th-2 or IL-23 (25 ng/ml), IL-21 (25 ng/ml), IL-6 (25 ng/ml), IL-1β (12.5 ng/ml), TGF-β (5 ng/ml), anti-IL-4 (5 μg/ml), anti-IL-12 (5 μg/ml) and anti-IFNγ (5 μg/ml) for Th-17 cell clones. At day 3, cells were removed from the plate and cultured with respective cytokines and neutralising antibodies including 20 U/ml IL-2 (except Th-17). Depending on cell growth, cells were split with fresh medium containing above cytokines and antibodies. In some cases, at day 7 cells were restimulated as described above. Lineage commitment was finally confirmed via expression of respective transcription factors and cytokines (T-bet, GATA-3, RORγ and IFNγ, IL-4, IL-17 for TH1, TH2 and TH17 clones, respectively).

Isolation of IFNγ(+)T-cells: At day 7, 1.5×10^6 iTreg were restimulated with coated anti-CD3 (4 μg/ml) and IL-2 (100 U/ml) for 3.5 h in a 24-well plate. We followed the manufacturer's recommended procedure (IFNγ enrichment and -secretion assay kit, (Miltenyi Biotec) to enrich IFNγ-expressing live cells from iTreg with a slight modification of the last step: PE-labelled IFNγ(+)cells were separated on cell-sorter instead of using anti-PE microbeads with purity greater than 98%. As quality control, immediately after cell-sorting the IFNγ(+) cells were analysed for IFNγ-expression by intracellular staining and RT-PCR. Flow cytometric sorted IFNγ -positive iTreg (IFNγ(+)iTreg were rested in IL-2 (50 U/ml) for 2–3 days before setting up functional assays.

MLR cultures: Flow cytometric sorted 2×10^5 Tresp (donor 1) were activated with 1×10^6 irradiated (30 Gy) allogeneic PBMC (donor 2) in a 24 well plate and fed with IL-2 for every two days. On day 9, cells were restimulated with allogeneic PBMC of donor 2 for 40 h. Immediately IFNγ(+)iTreg were separated as described above and rested in IL-2 (50 U/ml) for 2–3 days. Thereafter, the suppressive capacity of these "donor 2-educated" IFNγ(+)iTreg was tested in an MLR setting comparing their effect on autologous Tresp (donor 1) with irradiated donor 2 PBMCs versus donor 3 PBMCs.

Tonsillar T-cells: After informed consent, tonsils and blood were taken from patients undergoing tonsillectomy from the clinics for Head and Neck Surgery, University of Heidelberg. The study protocol was reviewed and approved by the local ethics committee. Briefly, fresh tonsils were minced and filtered through a 70-μm pore size nylon filter (BD Biosciences). The cell suspension was centrifuged over Ficoll–Hypaque. CD127(+)cells were collected using biotin-labelled CD127-Ab and anti-biotin microbeads (MACS-beads,