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IL-10/IFNγ co-expressing CD4$^+$ T cells induced by IL-10 DC display a regulatory gene profile and downmodulate T cell responses

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ABSTRACT

Induced regulatory T cells (iTreg) are imperative for tolerance induction and spreading of infectious tolerance. *Ex vivo* generated tolerogenic dendritic cells (tDCs) have strong therapeutic potential to induce antigen-specific iTreg. We previously demonstrated that IL-10 tDC-primed T cells are very suppressive and produce IL-10. Here, we show that the majority of IL-10$^+$ T cells co-express IFNγ, giving rise to the question whether these cells are proinflammatory or regulatory. Whole genome gene expression analysis revealed a strong regulatory gene profile and a suppressed Th1 gene profile for IL-10/IFNγ co-expressing CD4$^+$ T cells. Protein analysis confirmed an extensive regulatory phenotype for IL-10$^+$/IFNγ$^+$ T cells, with specific enhanced expression of GARP and PD-1. In line with these data, isolated IL-10$^+$/IFNγ$^+$ T cells displayed potent suppressive capacity. Thus, IL-10/IFNγ co-expressing CD4$^+$ T cells induced by IL-10 tDC show dominance of immunomodulation over Th1-mediated immunoactivation and can contribute to induction or spreading of immunological tolerance.

1. Introduction

Ex vivo generated tolerogenic dendritic cells (tDC) have the therapeutic potential to induce antigen-specific immunological tolerance in autoimmune diseases or in transplanted patients. We and other groups developed different protocols to culture tDC under GMP conditions (as reviewed by Schinnerling et al. [1]). tDC may induce peripheral tolerance by direct downmodulation of undesired effector T cell immunity [2–4] or indirectly by promoting development of induced regulatory T cells (iTreg) [5–8]. The latter pathway transfers tolerogenic potencies from tDCs to T cells and allows spreading and amplification of immune regulation. iTregs therefore form effective vehicles for infectious tolerance.

The phenotype of the tDC-primed T cells may vary depending on the type of tDC used [9–12]. One of the best studied iTregs are Tr1 cells, these Tregs are induced upon antigen-specific priming of naive T cells by tolerogenic DC-10 cells [13]. Tr1 cells produce large amounts of IL-10, TGFβ and IL-5, and variable amounts of IFNγ and suppress effector T cell proliferation through secretion of IL-10 and TGFβ [14,15]. Only recently it was elucidated that Tr1 cells can be identified and isolated based on co-expression of CD49b and LAG-3 [16].

Clearly, expression of IL-10 is not confined to Tregs. CD4 T effector cells are more plastic than previously thought [17] and IL-10 can be co-expressed by Th1, Th2 and Th17 cells [18]. IL-10 coproduction by Th1 and Th17 cells was suggested to mainly serve to protect against excessive immunopathology. Specifically in chronic infections, in human and in experimental animal models, CD4$^+$ T cells are found that produce high amounts of both IL-10 and IFNγ [19–23]. Indeed the IL-10 from these Th1 cells was demonstrated to play an important regulatory role for host protection [21,22]. These observations have made the distinction-line between the classical immunoactivatory CD4 T effector lineages and iTreg less clear. Also natural Tregs (nTregs) proved not to be a homogeneous population. By sensing environmental cues they can adjust their migratory and functional properties. This phenotypical plasticity of the nTregs is controlled by the same lineage-specific transcription factors that determine the effector Th cells they regulate, e.g. Th1-like nTregs expressing T-bet [24–26].

The recognition of plasticity between the various immunoactivatory and immunoregulatory CD4 T cell lineages makes it nowadays very important to clearly delineate the functional potential of CD4 effector T cells that co-express multiple lineage-specifying cytokines. We previously demonstrated that IL-10 tDC-primed T cells are very suppressive and produce IL-10, but that IL-10 is not the decisive regulatory molecule [27]. In this study we analyzed the primed T cells in more detail and found that the majority of the IL-10$^+$ T cell population co-expressed IFNγ. Whole genome gene expression analysis and protein expression analysis demonstrated that induced IL-10/IFNγ co-expressing T cells exhibited a strong regulatory phenotype, with enhanced expression of multiple regulatory proteins like GARP and PD-1 and downmodulation

Abbreviations: APC, antigen presenting cell; DC, dendritic cells; DP, double positive; IL-10, interleukin 10; mDC, mature DC; nTreg, natural Treg; SP, single positive; tDC, tolerogenic DC; Th cell, T helper cell; Treg, regulatory T cell.

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of typical Th1-like signature. Isolated IL-10$^+$/IFNγ$^+$ T cells displayed a potent suppressive capacity on responder T cell proliferation, demonstrating that the regulatory function of these cells is dominant compared to their immunoactivatory potential.

2. Materials and methods

2.1. Antibodies and reagents

CellGro DC serum-free medium, GM-CSF, IL-4, IL-1β and TNFα were all obtained from CellGenix (Freiburg, Germany). IL-10 and IL-2 were purchased from PeproTech (Rocky Hill, USA). PGE_2, PMA, ionomycin and brefeldin A were all obtained from Sigma-Aldrich (Steinheim, Germany). Penicillin and streptomycin were obtained from Gibco (Merelbeke, Beldium). For co-cultures Iscove's Modified Dulbecco's Medium (IMDM, Bio Whittaker, Verviers, Belgium) with 10% fetal calf serum (Bodinco, Alkmaar, The Netherlands) was used as described [28]. Adalimumab (Humira; Abbott) $F(ab)_2$ fragments were used as TNFα blocking agent at 10 μg/ml. As isotype control, $F(ab)_2$ fragments of an irrelevant monoclonal antibody directed against FelD1 (cat allergen) were used (Sanquin Reagents, Amsterdam, The Netherlands).

The following fluorochrome-labeled monoclonal antibodies were used: CTLA-4, Galectin-3, GARP, IFNγ and IL-4 from Becton Dickinson (BD Biosciences, San Jose, USA). IL-10 was obtained from Diaclone (via Sanquin Reagents), PD-1 from R&D Systems (Minneapolis, USA), IL-17 from eBioscience (Vienna, Austria), and LAG-3 from LSBio (LifeSpan Biosciences; Seattle, USA). The following antibodies were used with an appropriate secondary antibody: Galectin-10 (R&D) and Legumain (Santa Cruz Biotechnology; Heidelberg, Germany). Isotype-matched controls from Dako (Glostrup, Denmark), Sanquin Reagents or BD Biosciences were used. Secondary rabbit-anti-mouse antibody from Dako was used.

2.2. Isolation and culture of monocyte-derived dendritic cells

Monocytes were isolated from fresh aphaeresis material of healthy volunteers (after informed consent) by using the Elutra™ cell separation system (Gambro, Lakewood, USA). Monocytes were cultured at 1.5×10^6 cells/3 ml in 6 well plates (Nunc, Roskilde, Denmark) in serum-free CellGro medium supplemented with IL-4 (800 IU/ml), GM-CSF (1000 IU/ml), penicillin (100 IU/ml) and streptomycin (100 μg/ml). After 6 days, the immature DC were matured with IL-10 (40 ng/ml; 1 h pre-incubation) together with IL-1β (10 ng/ml), TNFα (10 ng/ml) and PGE_2 (1 μg/ml) for 2 more days to generate IL-10 tDC, as described previously [27]. Alternatively, DC were matured with IL-1β, TNFα and PGE_2 without IL-10 to generate mature immunoactivatory (m)DC.

2.3. T cell-dendritic cell co-culture and intracellular cytokine staining

Naive CD4$^+$CD45RA$^+$CD45RO$^-$ T cells were isolated as described previously [27]. Allogeneic IL-10 tDC were co-cultured with 1×10^5 CD4$^+$ naive T cells (1:5) in 96-wells flat-bottom plates (Nunc) for 13–15 days. Anti-TNFα or isotype control $F(ab)_2$ fragments (10 μg/ml) were added at start of co-culture. Fresh medium plus recombinant human IL-2 (10 U/ml) was added at day 7 of co-culture, and the cells were expanded for the next 6–8 days. Subsequently, T cells were restimulated with PMA and ionomycin (10 ng/ml and 1 μg/ml, respectively) for 5 h in the presence of brefeldin A (10 μg/ml). Production of IL-4, IL-10, IL-17 and IFNγ was detected by intracellular FACS staining on an LSRII flow cytometer and analyzed with FACS Diva software (BD Biosciences). For phenotyping, T cells were incubated with specific monoclonal antibodies or appropriate isotype-matched controls.

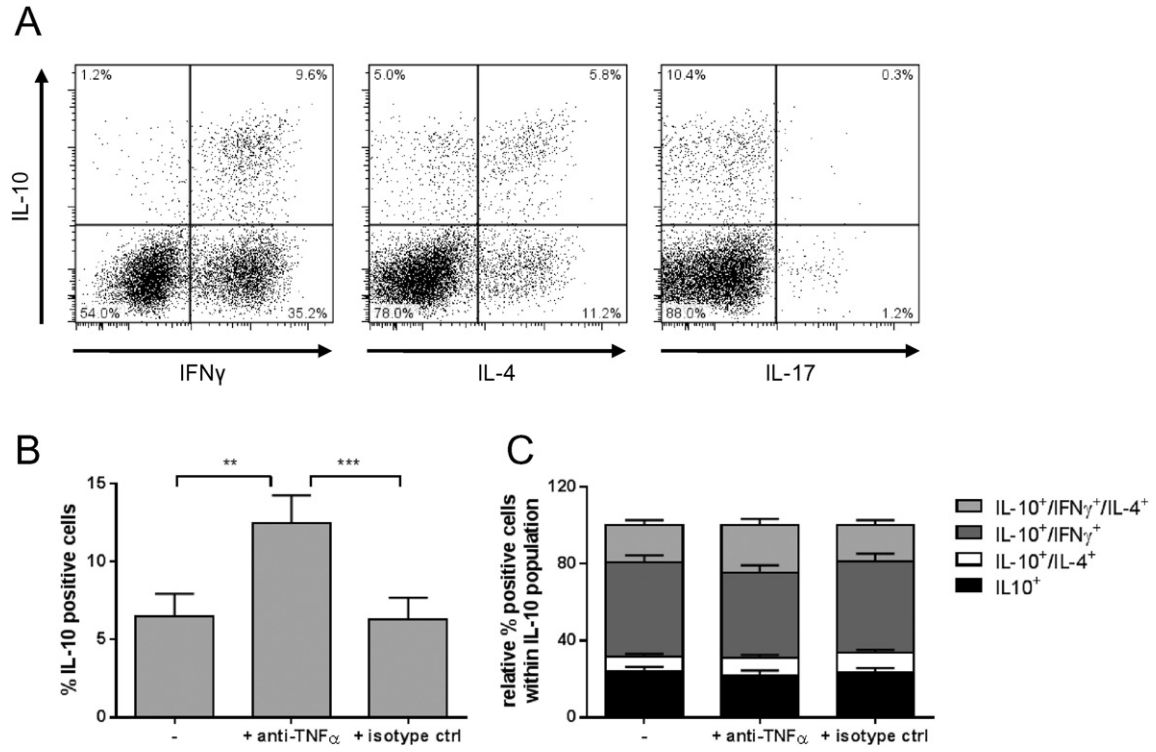

Fig. 1. IL-10 tolerogenic DC differentiate naive CD4$^+$ T cells toward IL-10 expressing cells that co-express IFNγ. Naive CD4$^+$ T cells were co-cultured with IL-10 tDC in the presence of anti-TNFα $F(ab)_2$. After 2 weeks, primed T cells were restimulated with PMA and ionomycin and stained intracellularly for IL-10, IFNγ, IL-4 and IL-17. (A) A representative dotplot of 14 independent experiments is shown. (B, C) T cells were primed by IL-10 tDC in the presence or absence of anti-TNFα $F(ab)_2$ or an isotype control $F(ab)_2$. Percentage of total IL-10 positive T cells (B) or relative percentage of IL-10 subpopulations (C) as determined by intracellular staining of restimulated primed T cells. Mean + SEM of 14 independent experiments is shown. The percentage of IL-10$^+$ T cells that co-expressed IFNγ was 71.3 ± 12.9%, 70.0 ± 12.9% and 66.4 ± 12.6% for T cells primed by tDC only, with additional anti-TNFα or with an isotype control, respectively. **$p \le 0.01$, ***$p \le 0.001$.