IN VITRO INDUCED CD8⁺ REGULATORY T CELLS INHIBIT SKIN INFLAMMATION

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CD8⁺ regulatory T cells appear impaired in number and/or function in some autoimmune diseases. However, the role of CD8⁺ regulatory T cells in the pathogenesis of skin inflammation and psoriasis remains unknown. In this study, we set out to analyze the capability of CD8⁺ regulatory T cells to inhibit skin inflammation in a murine model and to determine the frequency of CD8⁺ regulatory T cells in patients with psoriasis. We demonstrate that murine fully competent CD8⁺ regulatory T cells can be induced by stimulating naïve CD8⁺ T cells in the presence of TGF- β and retinoic acid (RA). Importantly, *in vitro* induced CD8⁺ regulatory T cells significantly suppressed skin inflammation *in vivo*. Furthermore, we found that the frequency of regulatory CD8⁺CD25⁺Foxp3⁺ T cells is decreased in peripheral blood but increased in lesional psoriatic skin of patients with psoriasis. Thus, our study suggests a previously unappreciated role of CD8⁺CD25⁺Foxp3⁺ T cells in skin disorders, and induction of these cells *in vitro* may be an effective immunotherapy for skin inflammation.

Keywords: skin inflammation, immune regulation, CD8⁺ T cells, immune modulation

Abbreviations: TH: T helper cells; Foxp3: forkhead box protein 3; GFP: green fluorescent protein; RA: retinoic acid; DC: dendritic cell; Sp: spleen; CFSE: carboxyfluorescein diacetate, succinimidyl ester; CHS: contact hypersensitivity; DNFB: 2,4-dinitro-1-fluorobenzene; PBMC: peripheral blood mononuclear cells; CD: cluster of differentiation; CTLA: cytotoxic T-lymphocyte antigen; GITR: glucocorticoid-induced tumor necrosis factor receptor; TGF: transforming growth factor; IL: interleukin; TNF- β : tumor necrosis factor- β ; IFN- γ : interferon- γ ; PASI: Psoriasis Area and Severity Index

Introduction

Regulatory T cells play a crucial role in inflammatory responses by inhibiting immune reactions. The most prominent population of regulatory T cells is CD4⁺, but CD8⁺ regulatory T cells also exist. The postnatal human thymus contains a defined population of suppressive CD8⁺ T cells. They express CD25, CTLA-4, GITR and Foxp3 (forkhead box protein 3) and suppress in a contact-dependent manner via CTLA-4 and TGF- β [1]. Interestingly, various phenotypes of induced CD8⁺ regulatory T cells seem to exist in humans and in experimental animals [2–7]. Here, the most prominent population is CD8⁺CD28[−] and suppressive via IL-10 secretion [8]. Furthermore, the induction of CD8⁺ regulatory T cells through the stimulation of the T-cell receptor mostly leads to the expression of Foxp3 in these T cells [3, 4, 9–11]. Most of the adaptive CD8⁺ regulatory T cells appear to suppress via a cell-contact-dependent mechanism.

Psoriasis is a chronic disease affecting skin in 2–3% of the general population. It is characterized by abnormal epidermal proliferation and chronic cutaneous inflammation [12, 13]. However, the etiology regarding the initial trigger for developing this disease and its pathogenesis is not un-

derstood in detail. Although several types of immune cells and various mechanisms have been implicated in the pathogenesis of psoriasis, the skin infiltrating activated T lymphocytes and the local overexpression of proinflammatory cytokines clearly have pivotal roles in initiating and maintaining the disease [14, 15]. In particular, CD4⁺ Th1 cytokines, such as TNF- β , IL-2 and -12, and IFN- γ have been shown to dominate the disease [16]. In addition, the Th17 subset of CD4⁺ T cells, with the expression of IL-17 and IL-23, has recently emerged as central for the disease [14]. In contrast to CD4⁺ T cells, the role of CD8⁺ T cells in psoriasis is less well defined. However, at least 80% of the T cells in psoriatic plaques are CD8⁺ [17]. Recent data demonstrate that these CD8⁺ T cells efficiently produce Th1-related cytokines TNF- β and IFN- γ as well as Th17-related cytokines IL-17, IL-21 and IL-22 [18]. With respect to psoriasis, studies on the role of CD4⁺ regulatory T cells have been performed. Sugiyama et al. reported that CD4⁺CD25⁺Foxp3⁺ regulatory T cells are dysfunctional in patients with this disease, both in peripheral blood and in the psoriatic skin [19]. In addition, reduced numbers of CD4⁺CD25⁺Foxp3⁺ regulatory T cells were detected in the peripheral blood of patients with psoriasis [20].

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In this study, we investigated the role of CD8⁺Foxp3⁺ regulatory T cells in skin inflammation. Therefore, we generated CD8⁺Foxp3⁺ T cells *in vitro* and investigated the potential of CD8⁺Foxp3⁺ T cells for the inhibition of skin inflammation in a murine contact hypersensitivity (CHS) model. In addition, we analyzed the expression of CD25 and Foxp3 in CD8⁺ T cells from the peripheral blood of patients affected by psoriasis. Thus, our study illustrates a previously unappreciated critical role of CD8⁺Foxp3⁺ T cells in controlling skin inflammation.

Results

In vitro induction of CD8⁺Foxp3⁺/GFP⁺ T cells

We recently established a protocol for the effective induction of murine CD8⁺Foxp3⁺ regulatory T cells *in vitro* [21]. To allow the separation of Foxp3⁺ cells from Foxp3⁻ T cells, we used Foxp3/green fluorescent protein (GFP) transgenic reporter mice, in which GFP expression accurately identifies the Foxp3⁺ T cell population. Polyclonal CD8⁺Foxp3⁻/GFP⁻ T cells from Foxp3/GFP mice were stimulated with α -CD3 alone or a mixture of α -CD3, TGF- β and retinoic acid (RA). As shown in Fig. 1, the stimulation of CD8⁺ T cells with α -CD3 or a combination of α -CD3 and RA did not induce the expression of Foxp3. In contrast, stimulation in the presence of TGF- β induced the conversion of a small population of CD8⁺Foxp3⁺ T cells (5.2%), and this conversion was further increased by the addition of RA (26.1%).

Fig. 1. TGF- β /RA-induced CD8⁺Foxp3⁺/GFP⁺ T cells. CD8⁺CD25⁻ splenic T cells from Foxp3/GFP mice were co-cultured with SP dendritic cells from BALB/c mice and soluble α -CD3 in the presence or absence of 2 ng/mL human rTGF- β and 100 nM retinoic acid (RA) for 4 days. Cells were stained for the expression of CD8 and Foxp3/GFP. The figure shows data representative of three independent experiments

TGF- β /RA-induced CD8⁺Foxp3⁺/GFP⁺ T cells exhibit suppressor function in vitro and in vivo

To analyze the suppressive potential of induced CD8⁺Foxp3⁺ T cells, we sorted CD8⁺Foxp3⁺/GFP⁺ and CD8⁺Foxp3⁻/GFP⁻ T cells after stimulation in the presence or absence of TGF- β /RA, as demonstrated in Fig. 2A. To check the inhibitory capability, the sorted T cells were co-cultured with carboxyfluorescein diacetate, succinimidyl ester (CFSE)-labeled CD4⁺CD25⁻ responder T cells in the presence of dendritic cells (DCs) and α -CD3 stimulation. At day 3 after stimulation, proliferation of responder cells was measured by the loss of CFSE dye. As summarized in Fig. 2B, TGF- β /RA-treated CD8⁺Foxp3⁺/GFP⁺ T cells markedly suppressed the proliferation of CD4⁺ responder T cells, which demonstrated the regulatory activity of CD8⁺Foxp3⁺ T cells *in vitro*.

In the next step, we examined the capability of TGF- β /RA-induced CD8⁺Foxp3⁺ T cells to inhibit skin inflammation. Skin painting with haptenizing and irritating agents causes CHS reactions that have traditionally been used as a model for psoriasis [22, 23]. Therefore, BALB/c mice were sensitized to 2,4-dinitro-1-fluorobenzene (DNFB), injected intravenously with CD8⁺Foxp3⁻/GFP⁻ or CD8⁺Foxp3⁺/GFP⁺ T cells, and ear-challenged later with DNFB. As expected, mice that were sensitized but challenged without the injection of any cells (positive control) exhibited a normal CHS response upon challenge (Fig. 3). Importantly, although the transfer of CD8⁺Foxp3⁻/GFP⁻ T cells slightly reduced the CHS response, the transfer of CD8⁺Foxp3⁺/GFP⁺ T cells resulted in a CHS response that was significantly lower than that of the positive control mice. This finding clearly illustrates the suppressive potential of TGF- β /RA-induced CD8⁺Foxp3⁺ regulatory T cells in skin inflammation.

Frequency of CD8⁺Foxp3⁺ T cells in the blood and lesional skin of patients with psoriasis

To investigate the potential role of CD8⁺Foxp3⁺ T cells in human skin inflammation, we analyzed the expression of Foxp3 in CD8⁺CD25⁺ T cells from the peripheral blood of patients with psoriasis compared to healthy control subjects. In healthy volunteers ($n = 10$), $23.6 \pm 3.7\%$ of CD8⁺CD25⁺ T cells co-expressed Foxp3. Interestingly, the percentage of Foxp3⁺ T cells within the CD8⁺CD25⁺ T cell population was significantly reduced in psoriatic patients ($n = 10$) with an average of $10.5 \pm 3.0\%$ (Fig. 4A). To characterize CD8⁺ T cells directly in lesional psoriatic skin in comparison to skin from healthy controls, serial sections of skin biopsies were labeled with fluorescence-labeled α -CD8 and α -Foxp3 and analyzed by fluorescence microscopy (Fig. 4B). Healthy skin biopsies demonstrated the presence of CD8⁺ T cells in the dermis. Among these, only very few cells co-expressed Foxp3. In contrast, numerous CD8⁺ T cells and CD8⁺Foxp3⁺ T cells were ob-

A **B**

Fig. 2. Immunosuppressive capability of TGF-β/RA-induced CD8⁺Foxp3⁺/GFP⁺ T cells. CD8⁺CD25⁻ splenic T cells from Foxp3/GFP mice were co-cultured as described in Fig. 1. For *in vitro* T-cell suppression assays, CD8⁺ T cells were separated into CD8⁺Foxp3⁻/GFP⁻ and CD8⁺Foxp3⁺/GFP⁺ T cells by FACS on CD8 and GFP expression (A), co-cultured with freshly isolated CFSE-labeled CD4⁺CD25⁻ responder T cells and SP DCs from BALB/c mice, and stimulated with soluble α-CD3. (B) Diagrams show the proliferation of responder T cells as determined by loss of CFSE dye and the inhibitory capability of TGF-β/RA-induced CD8⁺Foxp3⁺/GFP⁺ T cells

served in the dermis of psoriatic skin by direct immunofluorescence. The alteration in the frequency of CD8⁺Foxp3⁺ T cells in the blood and lesional skin of patients with psoriasis suggests a role of CD8⁺ regulatory T cells in skin inflammation.

Discussion

Psoriasis is an inflammatory skin disease, previously demonstrated to be driven by T cells. Therefore, studies on the phenotype of T cells in patients with psoriasis provide

Fig. 3. TGF- β /RA-induced CD8⁺Foxp3⁺/GFP⁺ T cells inhibit the effector phase of CHS. BALB/c mice were sensitized to DNFB, injected intravenously with sorted TGF- β /RA-treated CD8⁺Foxp3⁻/GFP⁻ or CD8⁺Foxp3⁺/GFP⁺ T cells, and ear challenged. As a negative control (neg. ctrl.), mice were not sensitized but were ear challenged, and as a positive control (pos. ctrl.), mice were sensitized and challenged without injecting any cells. Ear swelling was evaluated 36 h after challenge and is expressed as the difference between the challenged right ear and the unchallenged left ear. Data from two independent experiments are shown

valuable information about the interaction of regulatory and effector T cells *in vivo*. It was reported that CD4⁺ regulatory T cells are dysfunctional in patients with psoriasis [19]. We provide evidence that CD8⁺ regulatory T cells also play a role in the underlying immune pathogenesis of this disease. We could show for the first time that the percentage of these cells is reduced in the peripheral blood of patients with psoriasis but increased in the lesional psoriatic skin. We hypothesize that the recruitment of regulatory T cells from the peripheral blood to the site of inflammation is essential to control and eliminate inflammatory reactions. However, for CD4⁺ regulatory T cells it was demonstrated that these cells are functionally impaired in psoriasis [19], and as a result, they cannot restrain the ongoing inflammation. Once inflammation is initiated in psoriatic skin, an imbalance between anti-inflammatory and pro-inflammatory responses leads to a dysregulated activation of pathogenic cells.

In this study, we demonstrated that the *in vitro* treatment of naive CD8⁺ T cells with TGF- β and RA leads to the induction of CD8⁺Foxp3⁺ T cells with inhibitory capability *in vitro*. TGF- β is a multifunctional cytokine that controls proliferation, differentiation, and other functions in many cell types. More recently, the development of anti-inflammatory CD4⁺ and CD8⁺ regulatory T cells and the expression of their lineage-specific transcription factor Foxp3 have been shown to require TGF- β [10, 24], and new studies reveal that the development of pro-inflammatory Th17 cells also depends on TGF- β [25–27]. In contrast to the in-

Fig. 4. CD8⁺Foxp3⁺ T cells in the peripheral blood and lesional skin of patients with psoriasis. (A) PBMC from healthy donors and from patients with psoriasis were analyzed by flow cytometry for the expression of CD8, CD25 and intracellular Foxp3. Percentage of Foxp3⁺ T cells among CD8⁺CD25⁺ T cells and means $\pm$ SEM are shown. (B) Acetone-fixed cryostat sections (3 μ m) of normal human skin or lesional psoriatic skin were labeled with α -CD8 (green) and α -Foxp3 (red). CD8⁺Foxp3⁺ double positive (yellow) are denoted by arrows. Images are representative of three biopsies each. Scale bar = 50 μ m, magnification = $\times$ 400

hibitory effect of IL-6 on regulatory T-cell function [28], Th17 cell development requires IL-6 in addition to TGF- β [25]. Interestingly, increased levels of TGF- β 1 and IL-6 have been found in the epidermis and the serum of psoriasis patients [28, 29] and the TGF- β 1 serum level correlated with disease severity [30, 31], elucidating the increased level of Th17 effector cells in patients with psoriasis.

Vitamin A derivatives like RA are used for the topical therapy of psoriasis. RA binds to nuclear RAR- and RXR-receptors in keratinocytes and influences the proliferation, differentiation, and apoptosis of these cells. Actually, stud-

ies demonstrate an additional regulatory function of RA in immunological processes. Researchers have shown that RA inhibits Th17 polarization while activation of naive CD4⁺CD25⁻ T cells in the presence of TGF- β and RA *in vitro* further improves the TGF- β -mediated conversion into CD4⁺CD25⁺Foxp3⁺ regulatory T cells [32]. Interestingly, we could demonstrate that TGF- β /RA-induced CD8⁺Foxp3⁺ T cells exhibit regulatory capacity *in vitro* and suppress skin inflammation *in vivo*. Therefore, the treatment of psoriatic patients with RA may not only directly affect the keratinocytes but also block the expansion of Th17 effector cells and promote the induction and expansion of functional regulatory T cells.

In conclusion, we have detected a lower number of CD8⁺Foxp3⁺ T cells in the peripheral blood but an increased number in the lesional skin of patients with psoriasis than in healthy persons. Therefore, the *in vitro* generation of CD8⁺Foxp3⁺ regulatory T cells may provide a new therapy for psoriasis. We demonstrated that the *in vitro* stimulation of naive CD8⁺ T cells in the presence of TGF- β and RA leads to the induction of CD8⁺Foxp3⁺ regulatory T cells with the potential to suppress skin inflammation *in vivo*. The results presented here imply that therapies aiming at either increasing regulatory T cell numbers or specifically activating regulatory T cells could be beneficial in T-cell-mediated skin inflammations such as psoriasis.

Materials and methods

Patients

Ten patients (five females and five males) with severe psoriasis, defined by the severity index (PASI) ≥ 20 , and 10 healthy subjects (five females and five males) without any history or signs of skin diseases participated in this study after giving informed consent. All patients were not systemically treated for psoriasis for at least 2 years.

Immunofluorescence

Immunofluorescence staining was performed on acetone-fixed cryostat sections (3 μ m) of the normal human skin or the lesional skin from psoriasis vulgaris patients. Slides were incubated in the appropriate dilutions of antibodies (anti-human CD8, clone HIT8a and anti-human Foxp3, clone 150D/E4, both purchased from NatuTec, Frankfurt, Germany). Subsequently, slides were incubated with Alexa Fluor 488- or Alexa Fluor 594-labeled secondary antibodies (Invitrogen, Karlsruhe, Germany), and in some experiments, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Taufkirchen, Germany). Slides were examined using an Olympus BX61 microscope and the Analysis software (Olympus Soft Imaging Solutions, Münster, Germany).

Mice

Foxp3/GFP mice express both Foxp3 and GFP under the endogenous regulatory sequence of the Foxp3 locus and were obtained from Charles River Laboratories (Sulzfeld, Germany). BALB/c mice were obtained from Harlan (Harlan Winkelmann, Borcheln, Germany).

Isolation of lymphocytes

The autoMacs separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) was used for the isolation or depletion of lymphocyte subsets according to the manufacturer's instructions. CD4⁺ and CD8⁺ T cells were negatively selected with a purity of 95%.

Isolation of dendritic cells

For the isolation of DCs, splenic (SP) tissue was first cut into small pieces and then treated with 1 mg/mL collagenase type D (Roche Diagnostics GmbH, Mannheim, Germany) and 10 mg/mL deoxyribonuclease (DNase) I type II (Sigma-Aldrich Chemie GmbH, St. Louis, MO), diluted in phosphate buffered saline (PBS) containing 2% FCS and 2 mM EDTA, incubated for 45 min at 37 °C, and mechanically minced and filtered through a 100 μ m cell strainer. Cells were washed in PBS containing 2% FCS and 2 mM EDTA. CD11c⁺ cells were positively selected on MACS columns according to the manufacturer's instructions (Miltenyi Biotec).

Antibodies and flow cytometry

All antibodies were obtained from BD Biosciences (Heidelberg, Germany). Staining with α -Foxp3 (eBioscience, San Diego, CA) was performed according to the manufacturer's recommendations. Flow cytometric analysis was performed with a FACSCalibur flow cytometer and CellQuest software or with an LSR II and DIVA software (both from BD Biosciences).

In vitro induction of CD8⁺Foxp3⁺ T cells

For the induction of Foxp3 expression in polyclonal CD8⁺ T cells, 2.5×10^5 CD8⁺CD25⁻ naive T cells from Foxp3/GFP transgenic mice or human CD8⁺ T cells isolated from peripheral blood were stimulated with 0.5 μ g/mL soluble α -CD3, 2 ng/mL recombinant human TGF- β (R&D Systems, Wiesbaden-Nordenstadt, Germany), and 100 nM RA (Sigma-Aldrich, Germany). On day 2, 50 U/mL recombinant human IL-2 was added to the cultures. On day 4, Foxp3 expression in CD8⁺ T cells was determined by staining with α -CD8 and α -Foxp3 antibodies.

Suppression assay

For polyclonal T-cell suppression assays, TGF- β /RA-treated CD8⁺ T cells from Foxp3/GFP mice were separated into CD8⁺Foxp3⁻/GFP⁻ and CD8⁺Foxp3⁺/GFP⁺ T cells by fluorescence-activated cell sorting (FACS) on GFP expression, co-cultured with 1×10^5 freshly isolated CFSE-labeled CD4⁺CD25⁻ responder T cells in a 1:1 ratio and 0.5×10^5 Sp DCs from BALB/c mice, and stimulated with 0.5 μ g/mL soluble α -CD3 for 3 days.

Contact hypersensitivity

CHS experiments with BALB/c mice were performed as described elsewhere [33, 34]. Briefly, mice were sensitized to DNFB (Sigma-Aldrich, Germany) on day 0. On day 4, 2.5×10^6 sorted TGF- β /RA-treated CD8⁺Foxp3⁻/GFP⁻ or CD8⁺Foxp3⁺/GFP⁺ T cells were injected intravenously into each recipient mouse 24 h before elicitation of CHS responses. After 36 h, CHS responses were determined by the degree of swelling of the challenged right ear compared with the unchallenged left ear.

Statistical analysis

Statistical analyses were performed using Students's *t*-test, and values of $p \leq 0.05$ were considered significant.

Conflict of interest

The authors state no conflicts of interest.

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