Clinical Immunology 181 (2017) 51–59

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

$TCR^+CD3^+CD4^-CD8^-$ effector T cells in psoriasis

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ARTICLE INFO

Article history:
Received 3 May 2017
Received in revised form 14 June 2017
accepted with revision 14 June 2017
Available online 15 June 2017

Keywords:
Psoriasis
Programmed death-1
PD-1
Double negative T cells
IFN-γ
Effector memory T cells

ABSTRACT

The autoimmune/inflammatory disorder psoriasis is characterized by keratinocyte proliferation and immune cell infiltration of the skin. $TCR^+CD3^+CD4^-CD8^-$ "double negative" (DN) T cells can derive from $CD8^+$ T cells through the down-regulation of CD8. The inhibitory molecule programmed death (PD-)1 is expressed on activated T cells and plays a role in the maintenance of peripheral tolerance. A subset of DN T cells, characterized by the expression of PD-1, has recently been demonstrated to be self-reactive.
We demonstrate that a majority of DN T cells exhibits effector memory phenotypes, express IFN-γ, and fail to proliferate. DN T cells from psoriasis patients are characterized by reduced DNA methylation of the *IFNG* gene and increased PD-1 expression. Furthermore, PD-1 positive DN T cells infiltrate the epidermis in psoriatic skin lesions. Our observations offer additional insight into the molecular pathophysiology of plaque psoriasis and show promise as potential disease biomarkers and/or therapeutic targets for future interventions.

1. Introduction

Plaque-type psoriasis is a relatively common autoimmune disease characterized by increased keratinocyte proliferation and the infiltration of neutrophils, dendritic cells, macrophages, and T cells to dermal and epidermal layers. The role of effector $CD4^+$ or $CD8^+$ T cells, expressing a wide and somewhat stage-specific spectrum of pro-inflammatory cytokines (including IL-22, IL-17, IL-13, IFN-γ) has been established [1,2,3].

In response to antigen contact and subsequent activation, T lymphocytes proliferate and differentiate into effector or central memory T cells. Though most of our knowledge remains limited to the $CD4^+$ T cell compartment, all primed T cells either migrate to sites of inflammation or persist as circulating effector T cells. Effector $CD4^+$ and $CD8^+$ subsets are defined by their surface co-receptor expression patterns, which contribute to their homing characteristics [4]. Effector $CD8^+$ T cells were reported to be $CCR7^-$, and to express the inflammatory effector cytokine IFN-γ [4]. Because of their rapid effector function ex vivo, effector $CD3^+CCR7^-CD45RA^-$ T cells are generally referred to as effector memory (T_{EM}) cells [4]. Effector $CD3^+CCR7^-CD45RA^+$ T cells are terminally differentiated, and therefore referred to as terminal effector T cells (T_{TE}) [4]. Conversely, central memory T cells migrate to secondary lymphatic tissues, where they wait for a secondary challenge to exert enhanced immune responses [4,5]. Each T cell subset is characterized by specific cytokine expression patterns [6]. Unprimed, naïve T cells express a wide range of cytokines, including IL-2. Though effector memory subsets generally fail to express IL-2, they do express inflammatory effector cytokines, including Interferon-γ [7,4,6].

Effector T lymphocytes are central contributors to the inflammatory phenotype and tissue damage in various autoimmune/inflammatory disorders. Most data on T cell phenotypes and their contribution to tissue damage probably exists for the prototypical autoimmune disorder systemic lupus erythematosus (SLE). While the role of activated $CD4^+$ helper T cell subpopulation has been established in SLE, additional rare T cell subpopulations have more recently moved into the focus of research. Recent studies provide evidence that $TCR^+CD3^+CD4^-CD8^-$, so-called "double negative" (DN) T cells, can derive from $CD8^+$ T cells through the down-regulating of CD8 surface co-receptors [8]. With the loss of CD8, DN T cells acquire effector phenotypes capable of pro-inflammatory cytokine expression [9,10]. Several molecular mechanisms are involved in the process, including trans-repression of the *CD8A* and *CD8B* genes, and epigenetic remodeling of the extended *CD8* cluster though DNA methylation and histone modifications [10,8]. Increasing evidence supports the notion that DN T cells are involved in the pathophysiology of SLE [11,12,13]. Double negative T cells represent a relatively small and phenotypically heterogeneous T cell population. Based on their ability to express the pro-inflammatory cytokine IL-17A

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http://dx.doi.org/10.1016/j.clim.2017.06.002
1521-6616/

in SLE patients, DN T cells have been suggested to have effector phenotypes in autoimmune/inflammatory disorders [14,15]. Numbers of DN T cells are significantly increased in the peripheral blood of patients with SLE and they invade the kidneys of patients with SLE-associated nephritis [15], where they produce pro-inflammatory effector cytokines, activate T cells and induce immunoglobulin production [15]. Thus, DN T cells may play a central role in the pathophysiology and tissue damage in patients with SLE and perhaps additional autoimmune/inflammatory conditions [16] [17]. However, it remains somewhat controversial what proportion of DN T cells exhibit effector or regulatory T cell phenotypes and whether these phenotypes vary in healthy individuals and patients with autoimmune/inflammatory conditions, including psoriasis [18,15, 19,20,21,22,23].

The inhibitory co-receptor programmed death (PD-)1 is expressed on the surface of activated lymphocytes [24]. PD-1 plays a key role in maintaining peripheral tolerance by the suppression of T cell receptor signaling [25]. More recently, PD-1 moved into the focus of studies targeting peripheral tolerance and its disruption in autoimmune/inflammatory disease [26]. It became evident that PD-1 is expressed at increased levels on T lymphocytes from patients with autoimmune disorders, where it contributes to the limitation of inflammatory responses. Autoimmune disorders with increased PD-1 expression on T cells include SLE, psoriatic arthritis, and rheumatoid arthritis [27,28].

The aim of this study was to characterize DN T cells in controls and in patients with plaque-type psoriasis. To achieve this, frequencies of effector memory subsets ($CD3^+CD45RA^-CCR7^-$), terminal effector ($CD3^+CD45RA^+CCR7^-$), central memory ($CD3^+CD45RA^-CCR7^+$), and naïve or stem cell memory ($CD3^+CD45RA^+CCR7^+$) T cells were monitored. We also investigated Th1 cytokine expression patterns (IFN-γ), epigenetic requirements for cytokine expression, PD-1 expression as a potential marker of self-reactivity, and the recruitment of DN T cells to psoriatic skin lesions.

2. Material and methods

2.1. Study subjects and T cell culture

All psoriasis patients included in this study were diagnosed and recruited by the Division of Dermatology, Faculty of Medicine Carl Gustav Carus, TU Dresden, Germany. Matched healthy controls (blood donors) were recruited from the German Red Cross Blood Donor Service North-East. The study was reviewed and approved by the local ethics committee, and all included patients gave written informed consent.

Except for the initial PBMC FACS analysis, screening for effector memory T cells in controls vs. psoriasis patients (6/11 psoriasis patients received systemic treatment), all further experiments were carried out with blood samples from treatment-naïve patients with plaque-type psoriasis (supplement 1). *Psoriasis Area and Severity Index* (PASI) scores were evaluated at the time of blood sampling. Scores varied between 2.4 and 17.7 (mean 8.1 ± 5.8 SD).

Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral venous blood using Biocoll (Merck) and Leucosep Tubes (Greiner, Bio-One), following standard protocols. Untouched cytotoxic human $CD8^+$ T cells from patients and healthy controls were purified from PBMCs using the Human Total $CD8^+$ T Cell Isolation kit (Miltenyi Biotec). All primary human T cells were kept in RPMI 1640 medium supplemented with 10% FBS and 2 mM L-glutamine. For activation assays, $CD8^+$ T cells were incubated in the absence or presence of plate-bound anti-CD3 (OKT3; BioLegend) and anti-CD28 (CD28.2; BioLegend) antibodies for 120 h. All cell culture experiments were performed independently with T cells from single blood donors.

2.2. Flow cytometry analysis and fluorescence-activated cell sorting (FACS)

For flow cytometry and sorting, the following antibodies were used: Pacific Blue anti-CD4 (OKT4; BioLegend); Pacific Blue anti-human PD-1 (EH12.2H7; BioLegend); FITC anti-CD3 (OKT3; BioLegend); PE anti-CCR7 (G043H7; BioLegend); APC anti-CD45RA (HI100; BioLegend), APC-Cy7 anti-CD8 (SK1; BioLegend). Cells were stained with various fluorophore-conjugated antibodies in 2% BSA, 1 mM EDTA/PBS on ice for 30 min. Next, samples were measured on an LSR II flow cytometer (Becton Dickinson), data sets were analyzed with FlowJo software V10 (TreeStar). Sorting was performed using a FACSAria II cell sorter (Becton Dickinson) after 5 days with or without stimulation, by gating on viable $CD3^+$ and $CD4^-$, $CD8^{hi}$, $CD8^{lo}$, or $CD8^-$ T cell populations, as indicated.

Effector memory subsets were defined as $CD3^+CD45RA^-CCR7^-$ T cells, terminal effector as $CD3^+CD45RA^+CCR7^-$ T cells, central memory as $CD3^+CD45RA^-CCR7^+$ T cells, and naïve or stem cell memory cells as $CD3^+CD45RA^+CCR7^+$ T cells. In the $CD8^+$ compartment, effector T cells were defined as $CCR7^-$ [4]. In the $CCR7^-$ T cell subset, CD45RA expression was used to discriminate between differentiation states with distinct functional properties [29]. Generally, effector $CD3^+CCR7^-CD45RA^-$ T cells are referred to as effector memory (T_{EM}) [4]. Terminally differentiated effector $CD3^+CCR7^-CD45RA^+$ T cells are referred to as terminal effector T cells (T_{TE} also known as T_{EMRA} cells) [4].

2.3. Proliferation assay

T cells were cultured at a density of 10^6 cells/ml media and labeled with 5 μM CellTrace Violet dye (Thermo Fisher) according to manufacturer's instructions. Subsequently, cells were measured by flow cytometry after 5 days of culture in the absence or presence of stimulating anti-CD3 and anti-CD28 antibodies.

2.4. Isolation of genomic DNA and RNA

Sorted $CD8^{hi}$, $CD8^{lo}$, $CD45RA^+$ and $CD45RA^-$ DN T cells were collected, pelleted, and stored at −80 °C. Isolation and separation of genomic DNA and RNA from sorted cells was performed using ZR-Duet DNA/RNA MiniPrep kits (Zymo Research) according to manufacturer's protocol (including DNAse step for RNA isolation). For cDNA synthesis, iScript Reverse Transcription Supermix for RT-qPCR (BIO-RAD) was used following manufacturer's instructions.

2.5. Semiquantitative real-time PCR

Semiquantitative real-time PCR (qRT-PCR) was performed, using GoTaq qPCR Master Mix Real time PCR system (ProMega) according to manufacturer's instructions. Results were normalized to GAPDH and a calibrator of pooled human cDNA derived from sorted $CD8^+$ and $CD8^{lo}$ T-cells included on all PCR plates. Real-time PCR data were analyzed using the comparative C_T method [30]. Primer sequences for qRT-PCR are summarized in supplement 2.

2.6. DNA methylation qPCR

Mouse and human Interferon gamma genes (*IFNG*) were aligned, and conserved non-coding sequences (CNS) regions were determined (VISTA Genome Browser, available on the World Wide Web). CNS regions were defined as regions with sequence homology of >75% between the human and mouse genes over at least 200 base pairs. Based on the degree of conservation and the presence of methylation- sensitive restriction sites (MRSE sites), one region of interest was identified approximately 4.5 kilobases 5′ of the transcriptional initiation site of the *IFNG* gene (Fig. 3A). OneStep qMethyl kit (Zymo Research) was used as a real-time procedure for the determination of DNA methylation of specific sequences by MRSE digestion and quantitative polymerase chain reaction (PCR), following manufacturer's instructions. Human genomic DNA derived from sorted $CD45RA^+$ and $CD45RA^-$ DN T cells (as indicated in Fig. 3) was used as template. Primer sequences are summarized in supplement 2.