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*J Immunol* 2008; 181:1190-1198; doi: 10.4049/jimmunol.181.2.1190
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SOCS1 Regulates CCR7 Expression and Migration of CD4+ T Cells into Peripheral Tissues

Cheng-Rong Yu,* Rashid M. Mahdi,* Xuebin Liu,* Allen Zhang,* Tetsuji Naka,† Tadamitsu Kishimoto,† and Charles E. Egwuagu2*

Suppressors of cytokine signaling (SOCS) proteins control many aspects of lymphocyte function through regulation of STAT pathways. SOCS1-deficient mice develop severe skin and eye diseases that result from massive infiltration of inflammatory cells into these tissues. In this study, we have used SOCS1-, STAT1-, or STAT6-deficient mice, as well as, T cells with stable overexpression or deletion of SOCS1, to examine whether SOCS1 is involved in regulating lymphocyte trafficking to peripheral tissues. We show that SOCS1-deficient mice have increased numbers of T cells with characteristics of effector memory cells and expression of CCR7, a protein that promotes retention of T cells in lymphoid tissues, is markedly reduced in these cells. The decrease in CCR7 expression correlates with hyperactivation of STAT6, suggesting that aberrant recruitment of T cells into SOCS1-deficient mouse skin or eye results from abrogation of negative feedback regulation of STAT6 activation and CCR7 expression. Consistent with in vivo regulation of CCR7 expression and lymphocyte migration by SOCS1, forced overexpression of SOCS1 in T cells up-regulates CCR7 expression and enhances chemotaxis toward CCL19 or CCL21. CCR6 and CXCR3 are also up-regulated on SOCS1-deficient T cells and in situ analysis of the cornea or retina further reveal that these cells may mediate the chronic skin and eye inflammation through recruitment of Th1 and Th17 cells into these tissues. Collectively, these results suggest that SOCS1 regulates steady-state levels of chemokine receptors through its inhibitory effects on STAT pathways and this may underscore its role in regulating recruitment and retention of effector cells into nonlymphoid tissues. The Journal of Immunology, 2008, 181: 1190–1198.

Following positive and negative selection in the thymus, naive cells that populate the peripheral blood recirculate between the blood and secondary lymphoid organs in search of their cognate Ag. Their migration to and from these tissues is guided and regulated in part by interactions between chemokine receptors expressed on their cell surface and cognate ligands in tissues. Naive T cells and central memory T cells are retained in lymphoid tissues through their expression of the chemokine receptor CCR7 and interactions between CCR7 and cognate chemokine ligands, CCL19 and SCL (CCL21), expressed on high endothelial venules (1). On the other hand, upon encountering the cognate Ag, naive cells that differentiate into effector T cells or effector memory T cells lose expression of CCR7 and concomitantly acquire the ability to home to peripheral rather than lymphoid tissue. Although recruitment and migratory patterns of CCR7+ and CCR7− T cells have been described, mechanisms that regulate expression of CCR7 and those that promote the retention of memory and effector T cells in peripheral tissues are not well understood.

SOCS1−/− mice die within 3 wk after birth from massive infiltration of inflammatory cells into several peripheral T cell tissues, including the lung, pancreas, and heart (2, 3). Thus, the SOCS1-deficient mouse is an excellent model to study the role of SOCS proteins in regulating homoeostatic migration of lymphocyte and other inflammatory cells.

Suppressor of cytokine signaling (SOCS)1 is a newly described eight-member family of intracellular proteins that function as components of a negative feedback loop that regulate the initiation, intensity, duration, and quality of cytokine responses. The inhibitory effects derive from direct interactions between SOCS proteins and cytokine receptors that activate JAK/STAT pathways, leading to the recruitment of SOCS proteins to the signaling complex, their inhibition of the binding of STATs to tyrosine-phosphorylated cytokine receptors, and suppression of the catalytic activities of JAKs (8–11). SOCS proteins are now well established as important regulators of inflammatory processes, T cell development, and lineage commitment. SOCS proteins are differentially expressed in Th1 and Th2 cells and are implicated in Th cell lineage commitment and maintenance. Thus, recent studies showing that CXCR3, CCR5, and CCR7 are preferentially expressed by Th1 cell while Th2 cell preferentially expresses CCR3, CCR4, and CCR8 suggest that SOCS-mediated regulation of STAT pathways may be involved in differential regulation of chemokine receptor expression by T cells. In fact, SOCS proteins have been shown to regulate retention of hematopoietic stem cells in the bone marrow by blocking CXCR4− and SOCS1-deficient mice are characterized by massive infiltration of inflammatory cells into several peripheral T cell tissues, including the lung, pancreas, and heart (2, 3). Thus, the SOCS1-deficient mouse is an excellent model to study the role of SOCS proteins in regulating homoeostatic migration of lymphocyte and other inflammatory cells.

SOCS1−/− mice die within 3 wk after birth from massive infiltration of inflammatory responses into multiple organs and
unbridled IFN-γ signaling (4–6). However, although mice lacking both SOCS1 and IFN-γ or SOCS1 and STAT1 are rescued from premature mortality and significant morbidity (7, 8), mechanisms responsible for the aberrant recruitment of lymphoid cells into peripheral tissues are not well understood. In this study, we show that mice lacking both SOCS1 and STAT1 (double knock out (DKO)) develop severe eye and skin diseases that derive, in part, from abrogation of negative feedback regulation of STAT6 activation, resulting in down-regulation of CCR7 and up-regulation of CCR6, CXCR3 on the SOCS1-deficient Th cells, and aberrant recruitment of Th1 and Th17 cells into the eye and skin. Role of SOCS1 in regulating lymphocyte trafficking is further validated in studies of T cells with either overexpression or deletion of SOCS1.

Materials and Methods

Mice

The SOCS1−/−/STAT1−/−, SOCS1−/−/STAT6−/−, or STAT1−/− mice are on C57BL/6 background and have been described previously (7, 9). HNRP2-exon 7 (HNRE)-specific TCR transgenic mouse strain (a generous gift from M. Davis, Stanford University, Stanford, CA) has been described elsewhere (10). PCR primers used for genotyping are as follows: SOCS1, 5′-CACTCTCTCGCCACTCTCC-3′ and 5′-CAGCCT GTACATGCTGAA-3′; SOCS1/NeO, 5′-CGCACCAGCCGCAAA CATC-3′ and 5′-GAAAGCACTCAGGTGCAGCA-3′; STAT6, 5′-CAATGGGGAGAGCAAGGTTC-3′ and 5′-GGCTCTGAAATGAGCATG-3′; and STAT1, 5′-TGTAAGCTATGAGTGAGG C-3′ and 5′-TTGAGTACTGATGGAGT T-3′. The hybridization probes are: β-actin, 6FAM-CCATTCGTGCAGACATCC-5′ and 6FAM-TGGATGAGTCAGGAGGAC-3′; and CCR7, 6FAM-CGTCAACCCTTTCTTGTATGCCT-5′ and 5′-TTGATCGCGCTCCAGAAGAC-5′. For FACS analysis of inflammatory cells that infiltrate the eye and skin, enucleated eyes or minced skin tissues were digested in collagenase (10). For FACS analysis of cytokine and chemokine expression was detected using a BD Cytofix/Cytoperm kit (BD Biosciences) with requisite mAbs (eBioscience). FACS analysis was performed by the Core Facility at the University of Pittsburgh.

Histological analysis

Eyes were carefully dissected out, fixed in 4% glutaraldehyde for 30 min, and transferred to 10% buffered formalin. Specimens were dehydrated through graded alcohols and embedded in methacrylate. Serial transverse sections through the pupillary optic nerve plane were cut and stained with H&E. Photographs of representative sections were taken on a Zeiss photomicroscope as described previously (11).

Flow cytometry and intracellular cytokine staining

Freshly isolated thymocytes and lymphocytes isolated from the lymph node, spleen, peripheral blood, eye, or skin were stained with the following mAbs specific to: CD3, CD4, CD8, CD25, CD44, CD45RB, CD62L, CCR4, CCR5, CCR6, CXCR3, IFN-γ, IL-12, IL-17, IL-23, IL-33, LFA-1 or CD62L primers and probes were purchased from BD Biosciences. For intracellular cytokine staining, cells were stimulated with anti-CD3 Ab (10 μg/ml) for 4 h and the last hour with GolgiStop. Nuclear extract (5 μg) in binding buffer (20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14 M NaCl, 1 mM MgCl2, 10 mM KCl, containing the following protease inhibitors: 20 mM HEPES, 0.14
stored at −20°C until use. Samples were electrophoresed in 5% polyacrylamide gel in 0.25× Tris-borate-EDTA buffer and results were visualized by autoradiography.

**FIGURE 1.** *SOCS1*<sup>−/−</sup>*STAT1*<sup>−/−</sup> DKO mice exhibit severe ocular and skin diseases. A, Genotype analysis of tail DNA by PCR: lane 1, *SOCS1*<sup>+/+</sup>*STAT1*<sup>+/+</sup>; lane 2, *SOCS1*<sup>−/−</sup>*STAT1*<sup>−/−</sup>; lane 3, *SOCS1*<sup>−/−</sup>*STAT1*<sup>−/−</sup> mice; lane 4, *SOCS1*<sup>−/−</sup>*STAT1*<sup>−/−</sup>. B, Phenotypic analysis of peripheral lymphocytes by Western blotting: lane 1, WT; lane 2, *SOCS1*<sup>−/−</sup>*STAT1*<sup>−/−</sup>; and lane 3, *SOCS1*<sup>−/−</sup>*STAT1*<sup>−/−</sup>. C, Gross morphology of *SOCS1*-deficient mice (DKO) exhibiting age-dependent development of severe dermatitis. D, Histology of skin from normal mouse or *SOCS1*-deficient mice with chronic dermatitis and hyperkeratosis. E, Histology of 120-day-old DKO mouse eyes showing orbital cellulitis, retinal folds (.), chronic retinitis, or iritis. Arrows indicate inflammatory cells. H&E-stained sections.

**FIGURE 2.** *SOCS1* deficiency leads to accumulation of memory-like T cells with reduced levels of cell surface expression of CCR7. A and B, Freshly isolated thymocytes of lymphocytes isolated from the PBLs or lymph nodes of WT (*SOCS1*<sup>+/+</sup>*STAT1*<sup>+/+</sup>), STAT1 knockout (*SOCS1*<sup>−/−</sup>*STAT1*<sup>−/−</sup>), or DKO (*SOCS1*<sup>−/−</sup>*STAT1*<sup>−/−</sup>) mice were stained with Abs to CD3, CD4, CD8, CD25, CD45RB, CD62L, CCR6, CCR7, or CXCR3 and detected by four-color FACS analysis; plots are gated on CD3<sup>+</sup> and/or CD4<sup>+</sup> cells. C, Quantitative real-time RT-PCR analysis of lymph node T cell RNA from WT (bar 1), *SOCS1*<sup>+/+</sup>*STAT1*<sup>−/−</sup> (bar 2), or *SOCS1*<sup>−/−</sup>*STAT1*<sup>−/−</sup> (bar 3) mice.
chemotaxis in Transwell chemotaxis plates (15). Briefly, the cells were equilibrated at 37°C for 2 h. PBMC, cells were electroporated at 120 V, 0.2 cm gap for 20 ms (BTX 600; Genetronics). Stable transfection, Manassas, VA, was established by selection in 2 μg/ml D10.G4.1 Th2 cells (American Type Culture Collection) containing 2 × 10^{-5}/ml D10.G4.1 Th2 cells (American Type Culture Collection, Manassas, VA) was electroporated at 120 V, 0.2 cm gap for 20 ms using a square wave electroporator (BTX 600; Genetronics). Stable transfectants were established by selection in 2 μg/ml puromycin (Sigma-Aldrich).

In vitro chemotaxis assays

The chemotactic responses of the CD4+ T cells were performed as previously described (15). Briefly, the cells were equilibrated at 37°C for 2 h of chemotaxis in Transwell chemotaxis plates (5-μm pore size, Costar). The chemokines, EBV-induced molecule 1 ligand chemokine (ELC) (CCL19), or pBabe/anti-sense-SOCS3 plasmid DNA in culture medium (400 μl) containing 50% of the skin coat at 2 mo of age (Fig. 1C, left panel). By 4 mo of age, >95% of body hair is lost and the alopecia is accompanied by depigmentation of the entire body and ulcerations (Fig. 1C, right panel). Histological analysis of the skin of the DKO mice reveal marked infiltration of the tissues by inflammatory cells and development of chronic dermatisis and hyperkeratosis (Fig. 1D). In addition to the gross morphological manifestation of alopecia, the DKO mice develop cataracts and blepharosynechia (Fig. 1E). Histological analysis of the eye revealed massive infiltration of inflammatory cells into various tissues of the eye and these mice exhibit orbital cellitis and hallmark of chronic inflammation, including the development of retinal folds, chronic retinitis, and iritis (Fig. 1E).

Dysregulation of chemokine receptor expression correlates with the aberrant trafficking of lymphocytes into peripheral tissues of DKO mice

SOCS proteins have been shown to play a role in maintaining Th cells in a quiescent state and that transient inhibition of SOCS3 by Ag stimulation is essential in allowing activation of resting or naive T cells (16, 17). Consistent with these findings, we show here that a substantial percentage of the DKO CD4+ T cells has decreased levels of cell surface expression of IFN-γ signaling (4–6). To elucidate mechanisms responsible for the aberrant recruitment of lymphoid cells into peripheral tissues in SOCS1-deficient mice, we generated SOCS1−/−STAT1−/−DKO mice from SOCS1−/−STAT1−/− mice. Genotype of the DKO mouse strain was verified by tail DNA PCR analysis (Fig. 1A) and Western blot analysis (Fig. 1B). In contrast to the apparent normal histology of the lung, liver, and other organs of the DKO mice (data not shown), we show here that the DKO mice develop a severe progressive skin disease that results in marked alopecia with the loss of >50% of the skin coat at 2 mo of age (Fig. 1C, left panel). By 4 mo of age, >95% of body hair is lost and the alopecia is accompanied by depigmentation of the entire body and ulcerations (Fig. 1C, right panel). Histological analysis of the skin of the DKO mice reveal marked infiltration of the tissues by inflammatory cells and development of chronic dermatitis and hyperkeratosis (Fig. 1D). In addition to the gross morphological manifestation of alopecia, the DKO mice develop cataracts and blepharosynechia (Fig. 1E). Histological analysis of the eye revealed massive infiltration of inflammatory cells into various tissues of the eye and these mice exhibit orbital cellitis and hallmark of chronic inflammation, including the development of retinal folds, chronic retinitis, and iritis (Fig. 1E).

Results

SOCS1−/−STAT1−/− (DKO) mice exhibit severe skin and ocular diseases

SOCS1−/− mice die within 3 wk after birth from massive infiltration of inflammatory responses into multiple organs and unbriddled skin showing inflammatory infiltrates characterized by the presence of lymphocytes (L), macrophages (M), neutrophils (N), and eosinophils. D, Freshly isolated inflammatory cells from the skin or eyes of DKO mice were analyzed by cell surface flow cytometry or intracellular cytokine staining.

Generation of Th2 cells overexpressing sense or antisense SOCS1 or SOCS3 cDNA

Sense and antisense mouse SOCS1 cDNA fragments flanked by BamHI and EcoRI restriction sites were generated by PCR amplification from pEF-FLAG-1/SOCS plasmids provided by Dr. D. Hilton (Walter and Eliza Hall Institute, Melbourne, Australia). The fragments were directionally cloned into the BamHI and EcoRI sites of pBabe-Puro vector provided by Keith W. C. Peden (Laboratory of Retrovirus Research, Center for Biologics Evaluation and Research, Federal Drug Administration, Bethesda, MD) and sequence was verified by dsDNA sequencing (ABI 310 Genetic Analyzer; Applied Biosystems). For transfection, plasmid cDNAs were prepared by two cycles of CsCl banding. Five micrograms of pBabe (empty vector control), pBabe/SOCS1, pBabe/SOCS3, pBabe/antisense-SOCS1, or pBabe/anti-sense-SOCS3 plasmid DNA in culture medium (400 μl) containing 2 × 10^{-5}/ml D10.G4.1 Th2 cells (American Type Culture Collection, Manassas, VA) was electroporated at 120 V, 0.2 cm gap for 20 ms using a square wave electroporator (BTX 600; Genetronics). Stable transfectants were established by selection in 2 μg/ml puromycin (Sigma-Aldrich).

In vitro chemotaxis assays

The chemotactic responses of the CD4+ T cells were performed as previously described (15). Briefly, the cells were equilibrated at 37°C for 2 h of chemotaxis in Transwell chemotaxis plates (5-μm pore size, Costar). The chemokines, EBV-induced molecule 1 ligand chemokine (ELC) (CCL19), or pBabe/anti-sense-SOCS3 plasmid DNA in culture medium (400 μl) containing 2 × 10^{-5}/ml D10.G4.1 Th2 cells (American Type Culture Collection, Manassas, VA) was electroporated at 120 V, 0.2 cm gap for 20 ms using a square wave electroporator (BTX 600; Genetronics). Stable transfectants were established by selection in 2 μg/ml puromycin (Sigma-Aldrich).
CD62L\textsuperscript{high} (from 80.7 to 66.0%) and increased number of CD44\textsuperscript{high} cells (from 15.8 to 49.2%) (Fig. 2), suggesting that in the absence of SOCS1, unstimulated Th cells acquire features characteristic of effector memory cells. We further show that CCR7 expression is markedly reduced, particularly in the CD62L\textsuperscript{high} population of peripheral blood CD4\textsuperscript{T} cells (Fig. 2A). A similar decrease in CCR7\textsuperscript{T} cells is observed among lymph node CD4\textsuperscript{T} cells or thymocytes of the DKO (Fig. 2A). Since loss of CCR7 expression precedes acquisition of the ability of activated T cells to home to peripheral rather than lymphoid tissue, this result suggests that aberrant infiltration and retention of T cells in skin and eyes of DKO mice (Fig. 1, D and E) may derive from developmental defects in CCR7 expression. In addition to having elevated numbers of CCR7-negative peripheral T cells (Fig. 2A), we detected substantial numbers of activated CXCR3-expressing and CCR6-expressing lymphocytes in DKO mice (Fig. 2B). It is therefore of note that T cells that express low levels of CCR7 and higher levels of CXCR3 are associated with homing to inflamed skin tissues (18, 19), and expression of CXCR3 and CCR6 ligands (CCL20 and CXCL9, respectively) is up-regulated in corneal epithelia and keratocytes in mouse herpetic stromal keratitis (20). In addition, the \( \alpha_{4}\beta_{7} \) integrin that mediates trafficking to mucosal tissues is also up-regulated in DKO CD4\textsuperscript{T} cells (data not shown), further underscoring the role of SOCS1 in regulating trafficking of inflammatory cells to the eye. The regulatory effects of SOCS1 on CCR7 or CD62L expression is recapitulated at the RNA level as revealed by real-time PCR analysis of naive CD4\textsuperscript{T} cells isolated from lymph nodes of WT, STAT1-deficient, or DKO cells (Fig. 2C). In contrast to CCR7, expression of CXCR3 or CCR4 is elevated in DKO T cells, indicating selectivity of the effects of SOCS1 on chemokine receptors expression (Fig. 2C). LFA-1 expression is not perturbed in the DKO cells further, indicating that SOCS1 does not regulate expression of all proteins involved in recruitment of lymphocytes into tissues (Fig. 2C).

**CGR6\textsuperscript{T} Th17 cells are expanded in SOCS1-deficient mice**

Th17 cells have recently been implicated in the etiology of a number of human and mouse chronic inflammatory diseases including uveitis (intracellular inflammatory disease) and psoriasis (21, 22), and human Th17 cells are characterized by cell surface expression of CCR6 (23). It is therefore notable that freshly isolated (without antigenic stimulation) PBLs from DKO mice comprise substantial numbers of CCR6\textsuperscript{T} T cells that constitutively express IL-17 (Fig. 3A). Th17 cells are detected in both CD4\textsuperscript{T} and CD8\textsuperscript{T} compartments of the DKO mice. However, more of the Th17 cells are of the CD4\textsuperscript{T} cell phenotype, whereas a reciprocal increase in Th1 cells is observed in their CD8\textsuperscript{T} compartment (Fig. 3A), suggesting divergent roles of SOCS1 in regulating IL-17 and IFN-\( \gamma \) gene expression in CD4\textsuperscript{T} and CD8\textsuperscript{T} cells. Although SOCS1 is primarily considered as a negative feedback regulator of STAT1 signaling (24), analysis of lymph node cells of the DKO mouse strain shows that SOCS1 is also a potent inhibitor of IL-4, IL-6, IL-13, and IL-17 secretion (Fig. 3B) and suggests that elevated levels of these cytokines may contribute to the chronic inflammation observed in the DKO mice. The apparent discrepancy between expression patterns of IL-17 and IFN-\( \gamma \)-expressing DKO T cells detected by intracellular cytokine assay (Fig. 3A) are CD8\textsuperscript{T} cells (23% compared with 0.8% in the CD4 compartment) while more of the IL-17-expressing cells are CD4\textsuperscript{T} cells (9.6% compared with 4.2% in the CD8 compartment). In the ELISA, stimulation of the cells for 4 days may lead to disproportionate expansion of DKO CD4\textsuperscript{T} cells T cells that mainly produce IL-17 and not IFN-\( \gamma \). We also detected inflammatory cells in the eyes and skin of DKO but not in these
tissues of the WT mouse (Figs. 1 and 3C) and mRNA transcripts to IL-17A, IFN-γ, CCR6, MIP-3α (ligand for CCR3) were detected in retinas or cornea of DKO mice (data not shown). The predominant cell type detected are lymphocytes, interspersed with macrophages, neutrophils, and eosinophils (Fig. 3C). IL-17A, IFN-γ, or CCR6 mRNA transcripts were also detected and 20% or 13% of lymphocytes infiltrating the skin or eyes, respectively, are CCR6+ Th17 cells (Fig. 3D).

**SOCS1 induces CCR7 expression and chemotaxis in CD4+ T cells**

To determine directly whether SOCS proteins regulate the expression of CCR7 in T cells, we analyzed D10.G4.1 Th2 cells that constitutively express high levels of the SOCS1 or SOCS3 protein (16, 25). D10.G4.1 cells in which SOCS1 or SOCS3 is depleted by stable expression of antisense SOCS1 or antisense SOCS3 cDNA constructs were used as controls (16, 25). Consistent with published reports (26), RPA analysis on RNA from the stably transfected cells reveals that CCR5 and CCR2 are highly expressed in the AE7 Th1 while CCR4 is highly expressed in the D10.G4.1 Th2 cells. Interestingly, overexpression of SOCS3 induces a substantial reduction of CCR4 in the Th2 cells (Fig. 4A, right panel), suggesting that this chemokine receptor is under feedback regulation by this SOCS member. Of particular interest is the up-regulation of CCR7 by overexpression of SOCS1 in the D10.G4.1 cells (Fig. 4A, left panel). Specific up-regulation of CCR7 by SOCS1 is not restricted to increase in the transcription of the gene since expression of the CCR7 protein is also markedly elevated on the cell surface of D10.G4.1 cells with stable overexpression of the SOCS1 cDNA construct (Fig. 4B). To establish a functional role of SOCS1 in T cell trafficking, we examined chemotaxis response of SOCS1-overexpressing D10.G4.1 cells to the chemokine ELC (CCL19) or SLC (CCL21). Indeed SOCS1 induces migration toward its cognate ligands (Fig. 4C and D) and the response is dose dependent (Fig. 4D).

**Regulation of CCR7 in T cells is mediated by JAK-dependent mechanisms**

It is now a well-established concept that Th1, Th2, or Th17 cells are selectively recruited to sites of inflammation through differential expression of discrete chemokine receptors and that developmental commitment to any of these T cell lineages is dependent on the activation of JAK/STAT or MAPK pathways. We therefore used specific inhibitors that target either pathway to determine whether expression of CCR7 is dependent on genetic programs activated by JAK/STAT or MAPK-signaling pathways. CCR7 and CCR3 mRNA transcripts are readily detected in unstimulated cell cultures. Quantitative real-time RT-PCR analysis of CCR7 and CCR3 expression by unstimulated WT mouse naive T cells or cells stimulated with anti-CD3/anti-CD28. B. Primary lymph node T cells from WT mice were activated with anti-CD3/anti-CD28 Abs for 4 days in the absence or presence of chemical inhibitor of MAPK (PD98059; 10 μM), p38 MAPK (SB202190; 10 μM), or JAK (AG490; 50 μM) and were analyzed by FACS for cell surface expression of CCR7 or CD4. SOCS1 regulates CCR7 expression through its inhibitory effects on STAT6 pathways. C. Naive T cells from HEL TCR-transgenic mice were propagated for 4 days under Th0 (no Abs), Th1- or Th2-polarizing conditions and CCR7 mRNA transcripts were quantified by real-time RT-PCR. D. EMSA analysis of STAT6 activation in nuclear extracts (5 μg) prepared from lane 1, WT; lane 2, SOCS1+/− STAT1−/−; and lane 3 SOCS1−/− STAT1−/− T cells (E). Western blot analysis of STAT1 and STAT6 activation by mouse D10.G4.1 cells stably transfected with sense or antisense SOCS1 or SOCS3 cDNA expression vector (top panel); D10.G4.1 cells overexpressing SOCS1 or the empty vector were stimulated with IL-4 and analyzed for expression of pSTAT6 (bottom panel). F. Analysis of the relative abundance of CCR7-expressing thymocytes, unstimulated lymph node cells, or anti-CD3/anti-CD28-stimulated lymph node cells of WT and STAT6-deficient mice by FACS.

**FIGURE 5.** CCR7 expression in T cells is regulated by SOCS1 through its inhibitory effects onSTAT6 pathways. A. Quantitative real-time RT-PCR analysis of CCR7 and CCR3 expression by unstimulated WT mouse naive T cells or cells stimulated with anti-CD3/anti-CD28. B. Primary lymph node T cells from WT mice were activated with anti-CD3/anti-CD28 Abs for 4 days in the absence or presence of chemical inhibitor of MAPK (PD98059; 10 μM), p38 MAPK (SB202190; 10 μM), or JAK (AG490; 50 μM) and were analyzed by FACS for cell surface expression of CCR7 or CD4. SOCS1 regulates CCR7 expression through its inhibitory effects on STAT6 pathways. C. Naive T cells from HEL TCR-transgenic mice were propagated for 4 days under Th0 (no Abs), Th1- or Th2-polarizing conditions and CCR7 mRNA transcripts were quantified by real-time RT-PCR. D. EMSA analysis of STAT6 activation in nuclear extracts (5 μg) prepared from lane 1, WT; lane 2, SOCS1+/− STAT1−/−; and lane 3 SOCS1−/− STAT1−/− T cells (E). Western blot analysis of STAT1 and STAT6 activation by mouse D10.G4.1 cells stably transfected with sense or antisense SOCS1 or SOCS3 cDNA expression vector (top panel); D10.G4.1 cells overexpressing SOCS1 or the empty vector were stimulated with IL-4 and analyzed for expression of pSTAT6 (bottom panel). F. Analysis of the relative abundance of CCR7-expressing thymocytes, unstimulated lymph node cells, or anti-CD3/anti-CD28-stimulated lymph node cells of WT and STAT6-deficient mice by FACS.
lymphocytes and splenocytes and following activation in the presence or absence of the chemical inhibitor PD98059 (for MAPK), SB202190 (for p38 MAPK) or AG490 (for JAK), we observed that a requisite decrease in CCR7 expression that accompanies activation of naive T cells does not occur in AG490-treated cells, suggesting involvement of JAK pathways in the regulation of CCR7 expression (Fig. 5A). On the other hand, expression of CXCR3 is induced in cultures containing the MAPK inhibitor but not AG490 (Fig. 5A), suggesting that selective expression of chemokine receptors by T cells is differential regulated by activating distinct signaling pathways. We also examined the effects of MAPK and JAK pathways on the regulation of CCR7 expression at the protein level by FACS analysis. In naive CD4+ T cells used in this analysis, 83.5% (29.7 of 35.8) of the cells express CCR7 and following activation by anti-CD3/CD28 Abs expression of CCR7 is down-regulated to 8.9% (5.2 of 58.7) (Fig. 5B). However, following activation in the presence of AG490, the level of CCR7-expressing cells remains comparable to naive cells (90%) while significant diminution of CCR7-expressing T cells is observed in cultures containing PD98059, suggesting that activation-induced down-regulation of CCR7 expression is mainly dependent on JAK/STAT signaling pathways (Fig. 5B) and to a lesser extent on MAPK pathways.

**SOCS1 regulates CCR7 expression through its inhibitory effects on STAT6 pathways**

To further characterize JAK/STAT pathways that regulate CCR7 expression during T cell development, we stimulated naive T cells in culture medium containing cytokines that preferentially activate STAT1, STAT3, STAT4, and/or STAT6 and examined their effects on CCR7 expression. Naive T cells isolated from HEL TCR-transgenic mice were therefore stimulated under Th0 (no exogenous cytokine or Abs), Th1 (activation of STAT1, STAT4 by IL-12, and IFN-γ), or Th2 (activation of STAT6 by IL-4) polarization conditions. We show that CCR7 expression is up-regulated under the Th1-polarizing condition in which the Th6 pathway is inhibited (by anti-IL-4 Abs in the culture) but down-regulated under the Th2 condition with the functionally activated STAT6 pathway (Fig. 5C), suggesting that activation of STAT6 inhibits transcription of CCR7 in T cells. Since SOCS1 and SOCS3 regulate duration and intensity of STAT signals (16, 25, 27, 28), it was of interest to determine whether reduced expression of CCR7 in the SOCS1-deficient mice derive from loss of inhibitory effects of SOCS1 on STAT6 activation. Analyses of T cell lines with stable overexpression or deletion of SOCS1 or SOCS3 protein reveal that STAT6 pathway is constitutively activated in SOCS1-deficient T cells (Fig. 5D) while forced overexpression of SOCS1 selectively silences activation of STAT6 in T cells (Fig. 5E, top panel). The ability of IL-4 to induce activation of STAT6 is also compromised in cells with constitutive overexpression of SOCS1 (Fig. 5E, lower panel). In addition, we found that the CCR7 level is elevated in STAT6-deficient thymocytes or lymphocytes and TCR activation induces substantial reduction of CCR7 cell surface expression in WT lymphocytes compared with STAT6-deficient cells (Fig. 5F). Taken together, the results showing that inhibition of CCR7 expression in T cells derives, in part, from inhibitory effects of SOCS1 on STAT6-dependent pathways, provide mechanistic links between loss of SOCS1, aberrant migration of T cells to peripheral tissues, and development of chronic inflammation of the eye and skin. However, decrease in CCR7 expression observed in stimulated STAT6-deficient T cells suggests that negative regulation of other STAT pathways by SOCS1 may also contribute to regulation of CCR7 expression. In this context, it is of note that SOCS1 is also a negative regulator of CD3ζ- and Syk-mediated NF-AT activation (29), as well as other, signaling adaptor proteins such as VAV (30) that mediate T cell activation and these pathways may also contribute to regulation of CCR7 expression.

**Discussion**

In this study, we have shown that SOCS1/−/−/STAT1−/− mice are characterized by massive infiltration of inflammatory cells into the skin and eyes leading to the development of a psoriasis-like skin disease, severe alopecia, blepharitis, orbital cellulitis, iritis, retinitis, and retinal detachment. Substantial percentage of naive T cells of the DKO mice exhibit an activated phenotype, marked reduction of cell surface expression of CCR7 expression, and respond to cytokines in the absence of TCR stimulation. These results suggest that SOCS1 deficiency causes alteration in T cell differentiation and that SOCS1 may be involved in mechanisms that restrict mobility of naive lymphocytes to secondary lymphoid organs and also those that promote recruitment or retention of effector T cells in the skin and eye.

Accumulation of specific effector cells in inflammatory lesions or peripheral tissues such as the skin or eye derives from dynamic processes orchestrated in part by STAT-dependent selective expression of chemokine receptors on the different Th subsets. Although mechanisms by which STAT proteins regulate chemokine receptor expression is unknown, CXCR3, CCR2, CCR5, and CCR7 are expressed in Th1 cells (Figs. 3 and 4) while CCR3, CCR4, and CCR8 are expressed in Th2 cells (Fig. 4 and Ref. 31). These and other published reports (26) suggest that the preferential pattern of chemokine receptor expression in different Th cell types is coordinately regulated by lineage-specific development programs. However, unlike the dependency on STAT4 and STAT6 for differentiation into a Th1 or Th2 subset, respectively, acquisition of requisite chemokine receptor expression patterns does not require STAT4, suggesting additional levels of regulation. In this study, we have established that SOCS proteins are involved in regulating chemokine receptor expression and migration of CD4+ T cells. In addition to the marked reduction of cell surface expression of CCR7 on DKO T cells (Fig. 2), we have also shown that lymphocytes with forced overexpression of SOCS1 up-regulate transcription of CCR7. Markedly elevation of the CCR7 protein on the cell surface of D10.G4.1 Th2 cells with stable overexpression of the SOCS1 is noteworthy since expression of CCR7 is thought to be restricted to Th1 cells.

Retention of naive T cells in lymphoid tissues depends on CCR7 expression and its interaction with cognate chemokine ligands (CCL19 and CCL21) on high endothelial venules while homing of effector T cells to peripheral tissues is preceded by loss of CCR7 expression and acquisition of the ability to express CXCR3, CCR4, or CCR6 (1). In the DKO T cells, CCR7 expression is repressed while the expression of CXCR3 and CCR6 is up-regulated, suggesting that SOCS1 may function in vivo to promote the retention of naive cells in lymph nodes while repressing expressing chemokine receptors required for migration of effector cells to peripheral tissues. In addition to its role in regulating the intensity and duration of STAT signals, we show here that SOCS1 inhibits secretion of the proinflammatory cytokines IL-6 and IL-17 in vivo, and the dramatic increase in secretion of these cytokines by primary DKO T cells may account for the severe chronic skin and eye inflammation observed in DKO mice (Figs. 1 and 3C). It is important to note that IFN-γ- and IL-17-expressing cells are substantially expanded in DKO compared with WT or STAT1-knockout mice with a majority of the Th17 cells in the CD4 compartment while the IFN-γ-producing cells are predominantly in the CD8 compartment. It is however remarkable that >5-10% of freshly isolated (not subjected to Th17 polarization condition) CD4+ T
cells of the DKO mice are Th17 cells and a substantial percentage of them express the skin/eye homing receptor CCR6. Together, these results suggest that aberrant recruitment of CCR6+ or CXCR3+ T cells and their secretion of IL-17 and/or IFN-γ contribute to development of skin and ocular diseases in the DKO mice. These results are consistent with the recent implication of both Th1 and Th17 in the etiology of two potentially blinding chronic inflammatory diseases, scleritis or uveitis (21). A recent report showing that defect in Th17 differentiation in mice with conditional deletion of SOCS1 in CD4 T cells can be rescued by concomitant deletion of IFN-γ in T cells of SOCS1−/−/IFN-γ−/− mice (32) suggests that an increase in Th17 cells in DKO mice may derive in part from STAT1 deficiency in cells of the DKO mice. Surprisingly, the level of Th17 cells is 5-fold higher in DKO mice compared with STAT1-deficient mice (Fig. 3A), suggesting that the elevation of Th17 and Th1 cells in peripheral blood of the DKO mice cannot be wholly attributed to STAT1 deficiency in these mice. Moreover, the IL-6 level (Fig. 3B) and STAT3 activation (data not shown) are substantially elevated in the DKO compared with STAT1 knockout or WT mice, suggesting a potential role of both proteins in the observed increase of Th17 cells in DKO mice. In view of the increase of IL-13 secretion and profound skin inflammation in the inflammatory disease of the SOCS1 knockout mouse strain, the cytokine-induced CCR7 activation in T cells is inhibited by SOCS1 (Fig. 5E), and that cytokine-induced CCR6 activation in T cells is inhibited by SOCS1 (Fig. 5E), suggesting that the inhibition of CCR7 expression in T cells derives, in part, from the inhibitory effects of SOCS1 on STAT6-dependent pathways. Direct evidence for a functional role of SOCS1 in regulating T cell trafficking comes from chemokaxis assays showing that CCR7 can be induced to migrate toward cognate ligands (CCL19 or CCL21) by forced overexpression of SOCS1 (Fig. 4, C and D). It is of note that activation-induced down-regulation of CCR7 is blocked by the JAK inhibitor AG490 and to a much lesser extent by the MAPK inhibitor PD98059 (Fig. 5B), suggesting that mechanisms that regulate expression of CCR7 on naive and central memory T cells to restrict their trafficking to lymphoid tissues are dependent, in part, on JAK/STAT signaling pathways. On the other hand, expression of CXCR3 that enables homing of effector T cells to peripheral tissues is inhibited by the p38 kinase inhibitor SB202190, but up-regulated by PD98059. The differential sensitivity of CCR7 and CXCR3 to chemical inhibitors thus provides a rational basis for therapeutic targeting of these chemokine receptors and T cell trafficking.

In summary, the data presented in this report show: 1) T lymphocytes isolated from SOCS1−/− mice express lower levels of CCR7 and higher CCR6 and CXCR3 and distinctly contain higher amounts of Th17 cells in the CD4+ subset and higher IFN-γ-expressing cells in the CD8+ subset. 2) CD4+ T cells can be induced in vitro to up-regulate CCR7 expression and migrate toward its cognate chemokine ligands by forced overexpression of SOCS1. CCR7 is up-regulated in STAT6-deficient T cells and STAT6 activation is silenced in T cells by forced overexpression of SOCS1. Collectively, these observations suggest that SOCS1 regulates steady-state levels of CCR7 in T cells through its inhibitory effects on STAT6 signaling and underscores the role of negative feedback mechanisms orchestrated by SOCS1 in the recruitment and retention of effector cells in nonlymphoid tissues. The data presented thus establish mechanistic links between developmental activation of STAT pathways, SOCS expression, and regulation of chemokine receptor expression.

Acknowledgments

We thank An Amada-Obi and Hiroshi Takase (National Eye Institute, National Institutes of Health) for assistance with lympocyte isolation and RPA, respectively; Dr. Chi-Chao Chan (National Eye Institute, National Institutes of Health) for evaluating pathology slides.

Disclosures

The authors have no financial conflict of interest.

References


