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Differential activation of signal transducer and activator of transcription (STAT)3 and STAT5 and induction of suppressors of cytokine signalling in T_h1 and T_h2 cells

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Abstract

Cytokines direct the differentiation of naive CD4+ T cells into either IFN-γ-producing T_h1 cells or IL-4-producing T_h2 cells. In this study, we analyzed the activation of signal transducer and activator of transcription (STAT)1, STAT3 and STAT5 (together with STAT4 and STAT6), and the expression of the recently identified suppressor of cytokine signalling (SOCS) proteins, in differentiated T_h1 and T_h2 cells, both before and after re-stimulation with anti-CD3 and anti-CD28. In addition to the polarized activation of STAT4 in T_h1 cells and STAT6 in T_h2 cells, we found that STAT3 and STAT5 are selectively activated in T_h1 cells after differentiation. This activation of STAT3 and STAT5 was maintained after TCR re-stimulation. The selective activation of STAT3 and STAT5 in T_h1 cells was associated with differential induction of SOCS molecules. After re-stimulation, SOCS1 expression was significantly increased in T_h2 cells, but not in T_h1 and non-polarized 'T_h' cells. Additionally, the level of CIS was higher in T_h2 cells compared with T_h1 and T_h cells. In contrast, the expression of SOCS3 was higher in T_h1 cells. The differential induction of SOCS proteins was paralleled by the differential expression of cytokines in re-stimulated T_h1 and T_h2 cells (IFN-γ and IL-4/IL-13 respectively). Our results suggests that STAT3 and STAT5, possibly regulated by the SOCS proteins, may play a role in the differentiation of T_h cells, and in the maintenance of the T_h1 and T_h2 phenotype.

Introduction

Two CD4+ T_h subsets have been identified, based on their cytokine profile. T_h1 cells express IL-2, IFN-γ and tumor necrosis factor-β, whereas T_h2 cells express IL-4, IL-5 and IL-13 (1,2). These T_h subsets are essential for the regulation and optimization of immune responses against different types of pathogen. T_h1 cells participate in the cell-mediated immune response against intracellular pathogens, whilst T_h2 cells participate in the defence against extracellular pathogens (3). Overproduction of T_h1 cytokines has been implicated in organ-specific autoimmune diseases, such as multiple sclerosis. On the other hand, T_h2 cells are known to be involved in atopic and allergic responses (3,4). The

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differentiation of naive CD4+ T cells into T_h effector cells is regulated, mainly, by cytokines (5). IL-12 promotes T_h1 differentiation through the activation of signal transducer and activator of transcription (STAT)4 and mice lacking either IL-12 or STAT4 exhibit markedly reduced T_h1 responses (6). In contrast, IL-4 has been shown to drive differentiation towards the T_h2 phenotype by activating the STAT6 signalling pathway. Indeed, T cells from STAT6-deficient mice fail to develop into T_h2 cells (7). These T_h cell subsets cross-regulate each other through their prototypic cytokines (IL-4 and IFN-γ), promoting their own proliferation and differentiation in parallel with the suppression of the opposite T_h subset. IFN-γ down-regulates IL-4 receptor (IL-4R) expression, whereas IL-4 up-regulates its own receptor and down-regulates IL-12 receptor (IL-12R) expression (8,9). This receptor modulation is an important mechanism in determining the differentiation of T_h cells.

The identification of T_h1- and T_h2-specific transcription factors has led to a deeper understanding of the processes involved in committing T_h precursor cells to a particular fate. GATA3 and c-Maf are expressed in developing T_h2 cells, and have been shown to promote IL-4, IL-5 and IL-13 production, and inhibit IFN-γ production [reviewed in (10)]. GATA3 is believed to be induced by STAT6 and possibly affects the fate of T_h precursor cells through chromatin remodeling in the vicinity of cytokine genes (11–13). T-bet is specifically expressed in differentiating T_h1 cells and induces IFN-γ production, whilst also suppressing IL-4 and IL-5 production (14). A recent study has shown that T-bet is able to promote T_h1 differentiation independently of IL-12/STAT4, by both inducing chromatin remodeling of the IFN-γ locus and up-regulating IL-12R expression (15).

Differential expression of cytokine receptors and chromatin remodeling of cytokine genes are both mechanisms by which the T_h subset phenotypes are established and maintained. However, the repositioning of cytokine genes close to heterochromatin is not always associated with gene silencing (16). It has been shown that the IL-4R is present on fully differentiated T_h1 cells and that differentiated T_h2 cells likewise express the IFN-γ receptor. However, Huang *et al*. have shown that IL-4 signalling is impaired in T_h1 cells, with a corresponding reduction in the levels of IL-4 induced STAT6 and Janus kinase (Jak)3 phosphorylation (17). The mechanisms behind this impairment in cytokine signalling are not fully understood. It is known that STAT4 and STAT6 play a central role in T_h1 and T_h2 differentiation (respectively), and the activation of STAT1, STAT3 and STAT5 has been reported in studies of T_h1 associated autoimmune diseases, such as inflammatory bowel disease and rheumatoid arthritis (18–20) This, together with the observation that IL-12 induces STAT5 activation (21), suggests the possible involvement of these STAT molecules in T_h1 differentiation. STAT1, STAT3 and STAT5 are tightly regulated by suppressor of cytokine signalling (SOCS)1, SOCS3 and CIS; members of the SOCS family of proteins, which consists of eight members (CIS and SOCS1–7) (22). These SOCS proteins are inhibitors of cytokine signalling (23–25), which are induced by activated STAT proteins and inhibit STAT activation by competing with STAT proteins for binding sites on cytokine receptors and also by inhibiting the activity of Jak proteins. Thus, SOCS proteins participate in a classical negative feedback loop for cytokine signalling. Mice lacking the genes SOCS1, SOCS2 or SOCS3 have been generated, and all exhibit phenotypes associated with the deregulation of cytokine and hormone signalling (26–30). Collectively, these studies reveal an overlapping induction and function of the SOCS molecules, particularly between SOCS1 and SOCS3 (31).

Importantly, SOCS proteins are involved in inhibitory cross-regulation between different cytokine signalling pathways (32–34). IFN-γ has been shown to inhibit IL-4 mediated STAT6 phosphorylation in human monocytes, at least in part, by inducing expression of SOCS1 (35). Recent reports have also revealed a role for SOCS proteins in the differentiation of CD4+ T cells into T_h1 and T_h2 cells. It has been noted that IL-6 induces SOCS1 in activated CD4+ T cells and that SOCS1 prevents T_h1 differentiation by inhibiting IFN-γ-induced STAT1 activation (33).

In the present study, we examined the involvement of STAT1, STAT3 and STAT5, as well as SOCS1, SOCS3 and CIS, in the differentiation and maintenance of T_h1 and T_h2 cells. We found that STAT3 and STAT5 were selectively activated in T_h1 cells, both before and after re-stimulation, whilst their negative regulator (SOCS1) was induced in T_h2 cells. These data suggest that the STAT3 and STAT5 pathways may play a role in the regulation and maintenance of T_h1 cell differentiation.

Methods

Cell preparation

Spleens from B10.PL mice (H-2^u) bred at the School of Medical Sciences, Bristol University were used as a source of CD4+ T cells. Purified CD4+ T cells (>95% CD4+ as determined by FACS analysis) were obtained by positive selection using magnetic beads coated with anti-CD4 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

In vitro differentiation of T_h cells

For the generation of unpolarized 'T_h' cells, CD4+ T cells were cultured *in vitro* for 4 days with latex beads (Interfacial Dynamics, Portland, OR) coated with mAb to CD3 (145.2C11; BD PharMingen, San Diego, CA) and CD28 (37.51; BD PharMingen) as previously described (36), in the presence of rIL-2 (20 U/ml; R & D Systems, Minneapolis, MN). For the differentiation of T_h1 cells, rIL-12 (10 U/ml; R & D Systems) and anti-IL-4 mAb (11B11; 1 μg/ml; BD PharMingen) were also added to the culture. For the differentiation of T_h2 cells, rIL-4 (100 U/ml; R & D Systems) and anti-IFN-γ mAb (H22; 1 μg/ml; BD PharMingen) were added instead. After 4 days of differentiation, the T_h cells were thoroughly washed and rested in medium alone for 2 h before re-stimulation. The T_h subsets were re-stimulated using anti-CD3, anti-CD28-coated latex beads for 6 h.

Cytokine protein levels

Supernatants from the differentiation cultures were tested for IFN-γ and IL-4 content by specific ELISA according to the manufacturer's instructions (BD PharMingen). All cytokines were quantified with standard curves obtained with known amounts of recombinant mouse cytokines.

Quantitative transcript analysis

RNA was prepared from both non-stimulated purified CD4+ T cells and T_h cells stimulated with anti-CD3, anti-CD28-coated latex beads for 6 h or with rIL-4 (100 U/ml) for 30 min. Total RNA was extracted from purified CD4+ T cells using TRIzol (GIBCO, Rockville, MD) according to the manufacturer's instruction and was reversely transcribed using random hexamer primers (Amersham Pharmacia Biotech, Uppsala, Sweden). Quantitative real-time PCR was performed using a LightCycler (Roche Diagnostics, Mannheim, Germany) utilizing SYBR Green 1 (Roche Diagnostics) for the sequence-independent detection of DNA according to the manufacturer's instructions. In brief, reactions (20 μl) containing 2 μl cDNA, 3 mM $MgCl_2$, 150 μg/ml BSA (Roche Diagnostics) and 1 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) were denatured at 95°C for 5 min, followed by 35 cycles of amplification. Primer annealing temperatures ranged between 60 to 65°C. PCR primers included: CIS sense: 5′-TGGCTACTGCAGTGCACCTG-3′; CIS antisense: 5′-TCCAGCTGTCACATGCATGC-3′ (300 bp); SOCS1 sense: 5′- TTCTTGGTGCGCGACAGTCG-3′; SOCS1 antisense: 5′-ACGTAGTGCTCCAGCAGCTC-3′ (160 bp); SOCS3 sense: 5′-TGTGTACTCAAGCTGGTGCAC-3′; SOCS3 antisense: 5′-CATACTGATCCAGGAACTCC-3′ (310 bp), and GAPDH sense: 5′- CATTGACCTCAACTACATGG-3′; GAPDH antisense: 5′-GTGAGCTTCCCGTTCAGC-3′(500 bp).

The data was analyzed using the LightCycler Software 3 (Roche Diagnostics). CIS/SOCS transcript levels were normalized against GAPDH. The RNA preparations were also checked for genomic contamination and the specific signals obtained were 10-fold lower compared to the cDNA samples.

SDS–PAGE and western blot analysis

Total cellular lysates from T_h cells harvested after 4 days of differentiation (non-stimulated T_h cells) and T_h cells cultured in normal medium for 2 h and then re-stimulated anti-CD3 and anti-CD28 for 6 h (re-stimulated T_h cells) were made from 10×10^6 purified CD4+ T cells according to the method of Hibi *et al*. (37). Briefly, cells were lysed in 600 μl of cold lysis buffer consisting of 20 mM Tris (pH 7.7), 250 mM NaCl, 3 mM EDTA, 3 mM EGTA and 0.5% NP-40, to which protease and phosphatase inhibitors had been freshly added. After 30 min incubation on ice, the extracts were spun for 10 min in an Eppendorf centrifuge at 4°C to pellet cellular debris. The supernatants were removed and stored at –70°C.

Western blotting was conducted using standard techniques. Briefly, proteins from cellular lysates were separated on 10% SDS–PAGE NOVEX gels (Invitrogen, Carlsbad, CA). Following electrotransfer to supported nitrocellulose membranes (Invitrogen), blots were blocked with 5% non-fat dry milk in TBS/0.1% Tween for 1 h at room temperature. Blots were then incubated overnight at 4°C with the following antibodies: pSTAT3 (B-7) and pSTAT6 (Tyr641) from Santa Cruz Biotechnology (Santa Cruz, CA), pSTAT4 (Y693) from Zymed (South San Francisco, CA), and pSTAT1 (Tyr701) and pSTAT5 (Tyr 694) purchased from New England Biolabs (Beverly, MA). STAT1, STAT3, STAT4, STAT5, STAT6, Jak1 and Jak2 were form Santa Cruz Biotechnology, and pJak1 and pJak2 were form Affinity Bioreagents (Cambridge, UK). The blots were then washed with TBS/Tween (0.1%) followed by incubation with one of the following secondary antibodies: rabbit anti-mouse–horseradish peroxidase (HRP), swine anti-rabbit–HRP or rabbit anti-goat–HRP (Dako, Cambridge, UK). Finally the blots were developed using the ECL chemiluminescence detection kit (Amersham Pharmacia Biotech).

RNase protection assay

Cytokine-specific, ^{32}P-labelled (Amersham Pharmacia Biotech) RNA probes were generated through the *in vitro* translation (Riboquant Transcription Kit; BD PharMingen) of the mouse cytokine probe set mCK1 (BD PharMingen) according to the manufacturer's protocol. Total RNA, purified from anti-CD3 and anti-CD28 re-stimulated non-polarized T_h, T_h1 and T_h2 cells (10 μg/sample) or T_h subsets stimulated with IL-4 (100 U/ml) and IFN-γ (50 ng/ml) for 30 min, was hybridized overnight with the labeled cytokine probes using the Riboquant RPA kit purchased from BD PharMingen and subsequently RNase treated. Protected fragments were resolved on 5% PAGE gels.

Results

Induction of T_h1 and T_h2 differentiation in vitro

Purified CD4+ T cells were cultured under T_h1- and T_h2-polarizing conditions for 4 days. STAT activation and SOCS expression were analyzed before and after re-stimulation with anti-CD3 and anti-CD28. In order to confirm the T_h1 and T_h2 phenotype, both before and after the re-stimulation, the expression of T_h1- and T_h2-specific cytokines was analyzed by ELISA and RNase protection assay (Fig. 1A and B). The results show distinct patterns of cytokine expression between the different T_h subsets, which were consistent both before and after re-stimulation. IFN-γ was highly induced in the T_h1 population, but was barely detectable in the T_h2 population (Fig. 1A and B). In contrast, IL-4 and IL-13 were almost exclusively induced in T_h2 cells (Fig. 1A and B). A distinct cytokine profile was also detected in the non-polarized 'T_h' cells, which were cultured in the absence of any exogenous cytokines. These T_h cells expressed low levels of IL-2, IL-10 and IFN-γ, but no IL-4 or IL-13 (Fig. 1A and B). Expression of IL-10 by T_h1 cells was also observed, which is consistent with other recent reports (38–40). These data show that the T cells were polarized, and that the T_h1 and T_h2 phenotypes were maintained after TCR re-stimulation. To further confirm that the differentiation was stable, differentiated T_h1 and T_h2 cells were cultured in normal medium for 6 h and then stimulated with IFN-γ or IL-4 respectively. RNase protection assay results show that exogenously added IL-4 could not induce T_h2 cytokine production by T_h1 cells, neither could IFN-γ induce T_h1 cytokine production in T_h2 cells (Fig. 1C). Therefore, in the absence of polarization conditions, the phenotypes of the differentiated T_h1 and T_h2 cells were stably maintained.

Jak and STAT activation in T_h1 and T_h2 cells

STAT4 and STAT6 are key transcription factors in the regulation of T_h1 and T_h2 development respectively. However, the involvement of other STAT molecules in the induction and maintenance of T_h differentiation has not been

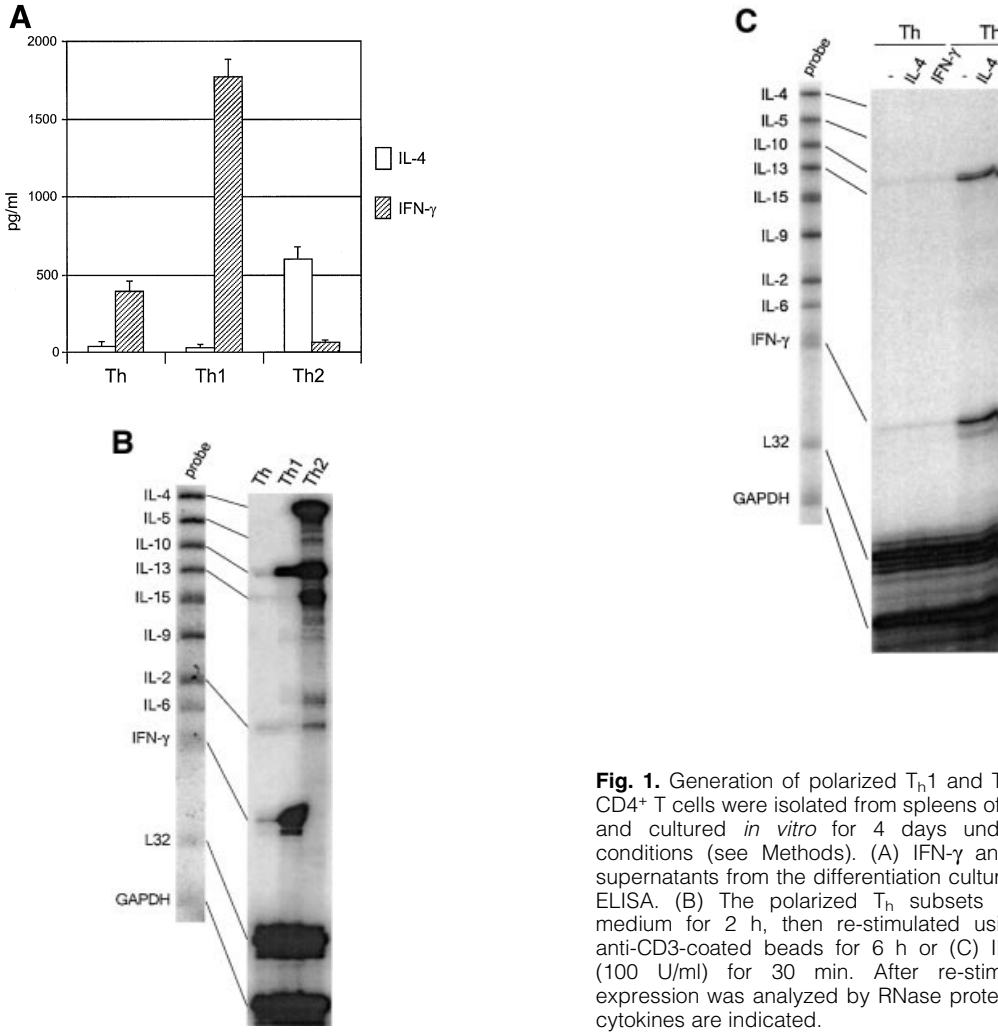

Fig. 1. Generation of polarized T_h1 and T_h2 subsets *in vitro*. Naive $CD4^+$ T cells were isolated from spleens of Tg4 TCR transgenic mice and cultured *in vitro* for 4 days under different T_h-polarizing conditions (see Methods). (A) IFN-γ and IL-4 were detected in supernatants from the differentiation cultures using cytokine-specific ELISA. (B) The polarized T_h subsets were cultured in normal medium for 2 h, then re-stimulated using either (B) anti-CD28, anti-CD3-coated beads for 6 h or (C) IFN-γ (50 ng/ml) and IL-4 (100 U/ml) for 30 min. After re-stimulation, cytokine mRNA expression was analyzed by RNase protection assay. The detected cytokines are indicated.

fully investigated. In order to determine which Jak proteins and STAT proteins are activated in T_h1 and T_h2 cells, total cell lysates from T_h1 and T_h2 cells, both before and after re-stimulation with anti-CD3/CD28 (see above), were analyzed by immunoblotting with antibodies specific to phospho-Jak or phospho-STAT proteins (Fig. 2).

STAT4 activation was selectively induced in T_h1 cells. In contrast, STAT6 activation was higher in T_h2 cells, both before and after re-stimulation with anti-CD3 and CD28 (Fig. 2A and B). This confirms the previous findings that STAT4 and STAT6 are involved in the regulation of T_h1 and T_h2 differentiation respectively (41). In addition to the reciprocal activation of STAT4 and STAT6 in T_h1 and T_h2 cells, the amount of STAT3 and STAT5 phosphorylation was significantly increased in T_h1 cells, but not in T_h2 cells, before and after re-stimulation with anti-CD3 and anti-CD28. This suggests that STAT3 and STAT5 activation in T_h1 cells is not due to the polarization pressure for T_h1 differentiation (Fig. 2A and B). Low STAT3 and STAT5 activation in T_h2 cells was associated with increased SOCS1 mRNA levels (see below). STAT1 was predominantly activated in T_h1 cells after polarization; however, all of the T_h subsets showed similar levels of STAT1 phosphorylation following re-stimulation (Fig. 2A and B).

Since STAT proteins are activated by Jak proteins, the differential activation of STAT proteins in the T_h subsets may result from the differential regulation of Jak proteins. For example, SOCS proteins can inhibit Jak/STAT signalling by interacting with and inhibiting the activation of the Jak proteins [reviewed in (42)]. However, despite the clear differences in STAT3 and STAT5 activation, activation of Jak1 and Jak2 was observed in all T_h subsets, although phosphorylation of Jak1 and Jak2 was slightly higher in T_h1 and T_h2 cells compared to non-polarized T_h cells (Fig. 3).

Induction of SOCS molecules in T_h, T_h1 and T_h2 cells

The data, thus far, have revealed that STAT3 and STAT5 are activated in T_h1, but not T_h2, cells, and that this selective activation is maintained after TCR re-stimulation. One of the possible mechanisms behind this selective activation of STAT3 and STAT5 is the selective induction of negative regulators for Jak/STAT signalling pathways in T_h1 and T_h2 cells. The SOCS family members are induced by a large variety of stimuli and are potent inhibitors of Jak/STAT signalling (42). In order to investigate the association of SOCS expression and the selective induction of STAT activation in T_h1 cells, a real-time, quantitative RT-PCR assay

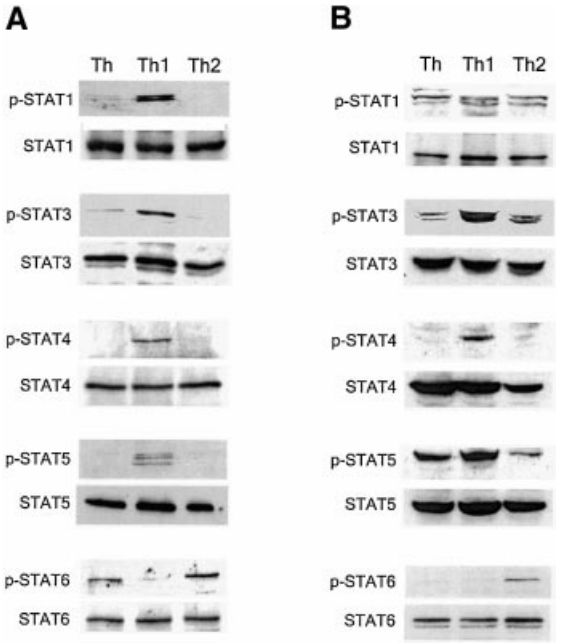

Fig. 2. Phosphorylation of STAT proteins in T_h subsets before and after TCR re-stimulation. Naive $CD4^+$ T cells were cultured under T_h1- and T_h2-polarizing conditions for 4 days. Western blot analysis of STAT activation was performed on cellular lysates prepared from (A) freshly differentiated T_h subsets and (B) T_h cells re-stimulated for 6 h using anti-CD3/CD28 antibodies. The results represent one of similar results from three independent experiments.

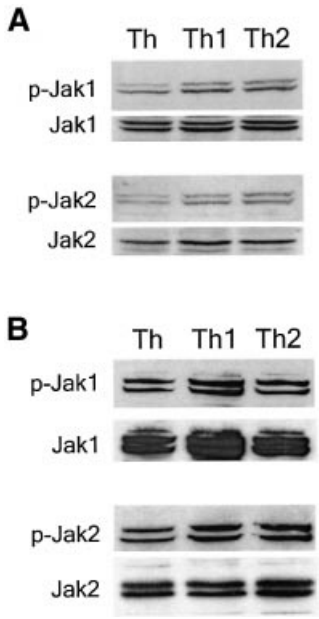

Fig. 3. Western blot analysis of Jak phosphorylation in T_h, T_h1 and T_h2 cells. Cell lysates from differentiated T_h cells re-stimulated with anti-CD3/CD28 antibodies for 6 h were resolved on 10% SDS–PAGE gels and transferred to nitrocellulose membranes. The activation of Jak1 and Jak2 was examined using phospho-Jak1- and -Jak2-specific antibodies. The results represent one of similar results from three independent experiments.

was used to examine the SOCS expression profiles of both T_h1 and T_h2 cells, before and after re-stimulation with anti-CD3/CD28. No induction of SOCS mRNA was detected in differentiated T_h1 and T_h2 cells before re-stimulation (data not show), in line with another recent report (17). However, following TCR re-stimulation, the level of SOCS1 mRNA was highly induced in T_h2 cells, compared to T_h and T_h1 cells (Fig. 4A). In addition to the induction of SOCS1 mRNA, the level of CIS mRNA was also increased, to some extent, in T_h2 cells compared to T_h1 cells (Fig. 4A). SOCS1 and CIS are inhibitors of the STAT5 signalling pathway (43,44). Furthermore, SOCS1 is a negative regulator of STAT3 signalling (25). Thus, the induction of SOCS1 and CIS in T_h2 cells may result in the suppression of STAT3 and STAT5 activation after TCR stimulation, facilitating maintenance of the T_h2 phenotype. In contrast, T_h1 cells showed slightly increased levels of SOCS3 mRNA (Fig. 4A). These data reveal a difference in SOCS expression between the subsets of T_h cells, which may help to explain the differential activation of STAT proteins observed.

In order to examine the involvement of IL-4 in the induction of SOCS1 expression in T_h2 cells, differentiated T cells were washed and then cultured with or without IL-4 or with anti-CD3 and anti-CD28. Interestingly, culturing T_h2 cells in the presence of IL-4 did not affect the expression of SOCS1, compared to media alone. However, when cultured in the presence of anti-CD3 and anti-CD28, a significant increase in SOCS1 expression was observed (Fig. 4B). TCR re-stimulation did not induce expression of SOCS1 in T_h1 cells. These data suggest that the selective induction of SOCS1 in T_h2 cells is first established during T_h2 differentiation and is then induced upon TCR stimulation. It is possible that SOCS1 expression, induced by TCR re-stimulation, could prevent the TCR-induced expression of IFN-γ in T_h2 cells. IFN-γ induces SOCS1 expression as a feedback inhibition mechanism. However, SOCS1 expression in T_h1 cells was low, despite the detection of high levels of IFN-γ mRNA (Fig. 4A).

Discussion

It is well established that T_h cell differentiation is induced by distinct cytokine signalling pathways [reviewed in (45)]. However, the mechanisms resulting in the inhibition of T_h2 cytokine production in T_h1 cells and T_h1 cytokines in T_h2 cells are not entirely clear. TCR stimulation of recently differentiated T_h2 cells leads to the production of IL-4, which is neither influenced by the cytokine environment nor dependent on IL-4 signalling (46–48). On the other hand, IFN-γ expression by differentiated T_h1 cells appears to be dependent on the cytokines present during re-stimulation (48). Although the selective expression of IFN-γ in T_h1 cells and IL-4 in T_h2 cells may be regulated by non-cytokine-mediated transcription factors, such as T-bet in T_h1 and GATA3 in T_h2 (10), it has been shown that the endogenous production of IL-4 by T_h2 cells renders them unresponsive to IL-12 stimulation. However, if the IL-4 is neutralized in T_h2 cells they do become responsive to IL-12, following treatment with IFN-γ. This leads to the subsequent, endogenous, production of IFN-γ. IL-4, therefore, appears to function to stabilize the T_h2 phenotype (47).

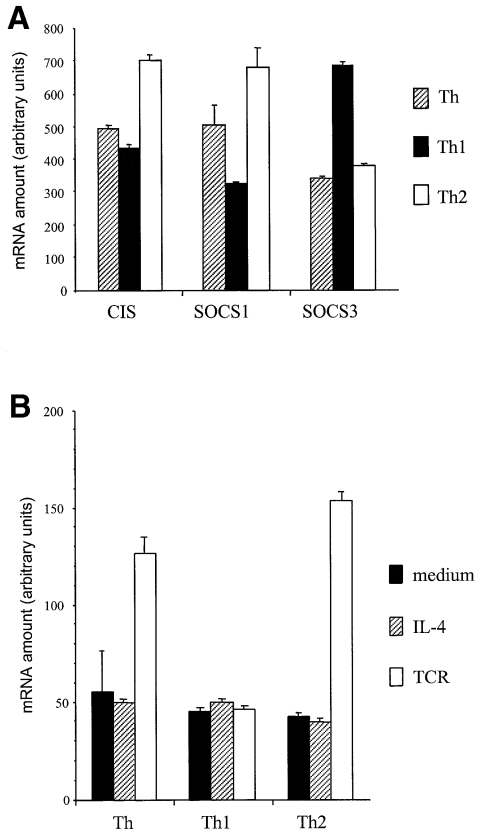

Fig. 4. (A) Expression of CIS, SOCS1 and SOCS3 molecules in re-stimulated T_h, T_h1 and T_h2 cells. Total RNA were isolated from the different T_h subsets following re-stimulation with anti-CD28 and anti-CD3 antibodies for 6 h. The expression of CIS, SOCS1 and SOCS3 was determined by quantitative real-time PCR. The SOCS expression levels were normalized against the housekeeping gene GAPDH. The presented results are mean values of five different experiments. (B) Expression of SOCS1 in T_h2 cells is induced in response to TCR stimulation, but not to IL-4. T_h, T_h1 and T_h2 cells were differentiated for 4 days, and were then allowed to rest in normal medium for 2 h. The T_h subsets were then stimulated with either IL-4 (100 U/ml) or anti-CD3/anti-CD28 antibodies, or left in medium alone for 6 h. The expression of SOCS1 mRNA was assessed by real-time quantitative PCR. The presented results are the mean values of three experiments.

IL-12/STAT4 and IL-4/STAT6 are the key signalling pathways in the development of T_h1 and T_h2 subsets respectively. However, both IL-4 and IL-12 can also induce activation of STAT5 in T cells (49,50). In this study, we have shown that STAT5 is induced in T_h1 cells after polarization with IL-12 and anti-IL-4, but not in T_h2 cells following polarization by IL-4 and anti-IL-12. STAT5 activation is associated with the production of T_h1 cytokines, which suggests that the regulation of T_h1 development may not be entirely dependent on STAT4. Indeed, IL-12, via the high-affinity receptor IL-12Rβ1/IL-12Rβ2, induces the activation of Jak2 and Tyk2 (51), which are known to phosphorylate STAT5. Interestingly, IL-12-mediated STAT5 activation is inhibited by IL-4, but augmented by IFN-γ (51). However, although these results suggest a role for STAT5 in T_h1, but not T_h2, cell differentiation, there is no direct evidence to support this. The suppression of IL-4-induced STAT5 activation in T_h2 cells may be important to prevent the expression of T_h1 cytokines. Most interestingly, the selective activation of STAT3 and STAT5 in T_h1 cells was observed not only after polarization, but also following TCR mediated re-stimulation, in the absence of IL-12 and anti-IL-4. This suggests that T_h1 associated STAT3 and STAT5 activation is dependent on T_h1 cell differentiation, rather than the direct action of IL-12 on differentiated cells. However, our data does not explain what stimuli trigger the activation of STAT3 and STAT5 in T_h1 cells. A possible candidate could be IL-27, a recently discovered cytokine that has been implicated to play a role in the generation of T_h1 responses (52). However, it is still possible that STAT3 activation in T_h1 cells may result from IL-12 stimulation (41). Interestingly, although IL-10 is induced in both T_h1 and T_h2 cells, IL-10-mediated STAT3 activation could not be detected in T_h2 cells. Thus, STAT3 activation in T_h1 may also be dependent on T_h1 cell differentiation.

In their studies of T_h differentiation in STAT5$^{-/-}$ mice, Kagami *et al.* (53) observed that T_h2 development is impaired in STAT5 knockout mice, despite these mice showing normal IL-4-induced activation of STAT6. However, this impaired T_h2 development does not result directly from the lack of STAT5 in T_h2 cells, but from the impaired function of regulatory CD4$^+$CD25$^+$ T cells, which modulate the development of T_h2 cells (53). In normal mice, depletion of the CD4$^+$CD25$^+$ regulatory T cells leads to a decrease in T_h2 cell differentiation, suggesting that STAT5 might be involved in the development of CD4$^+$CD25$^+$ regulatory T cells (53). Together with our results, this indicates that STAT5 is not directly involved in T_h2 cell differentiation.

SOCS proteins are negative feedback regulators of the Jak/STAT signalling pathway, and can thus inhibit signalling by many cytokines, growth factors and hormones (42). Expression of SOCS1 and SOCS3, the best studied of the SOCS molecules, has been reported to be induced by almost all cytokines investigated (42). However, despite the distinct phenotypes seen in SOCS1 and SOCS3 knockout mice, the functional differences of these molecules are largely unknown. A possible role for SOCS1 in T_h cell polarization *in vivo* has recently been reported (54). This study showed a high level of IFN-γ and IL-4 expression in SOCS1$^{-/-}$ CD4$^+$ T cells after stimulation *in vitro* with anti-CD3 antibody (54). It is, therefore, possible that SOCS1 may play a role in the suppression of T_h1 cytokines in T_h2 cells and/or T_h2 cytokines in T_h1 cells. Mice deficient in SOCS1 suffer from lymphocyte-dependent multi-organ diseases and perinatal death. However, this can be rescued using lymphocytes from either SOCS1/STAT1 or SOCS1/STAT6 double-knockout mice (34), suggesting that SOCS1 negatively regulates both the IFN-γ/STAT1 and IL-4/STAT6 signalling pathways. The over-activation of IL-4/STAT6 and IFN-γ/STAT1 signalling in SOCS1$^{-/-}$ CD4$^+$ T cells also suggests a possible role for SOCS1 in the maintenance of T cell homeostasis rather than the regulation of T_h differentiation. We have shown here that SOCS1 mRNA is increased in T_h2 cells, compared with T_h1 cells, after re-stimulation with anti-CD3 and anti-CD28 antibodies. The role of SOCS1 in T_h2 cells may be different to that in undifferentiated T cells, because, in addition to the above, SOCS1 expression is associated with the expression of T_h2 cytokines and IL-4-induced STAT6 activation in T_h2 cells. There is also a correlation between

SOCS1 expression and the suppression of STAT3 and STAT5 in T_h2 cells.

SOCS1 is a strong inhibitor of IFN-γ signalling (55). In SOCS1-deficient mice, IFN-γ-induced STAT1 activation is much higher than in wild-type mice (34). However, despite the TCR-mediated induction of SOCS1 in T_h2 cells, the activation of STAT1 is similar in all T_h cell groups, indicating that the function of SOCS1 in T_h2 cells is likely to be associated with the suppression of STAT3 and STAT5 rather than the inhibition of STAT1 activation. CIS also has the capacity to inhibit signalling pathways in which STAT5 participates. Indeed, STAT5 has been shown to induce CIS protein expression. The high level of CIS expression seen in T_h2 cells correlates well with the low level of STAT5 phosphorylation we have detected in our system. These observations are in line with findings in the CIS-transgenic mouse. Mice overexpressing CIS exhibit enhanced T_h2 cell differentiation, in parallel with a decrease in T_h1 cells (44).

SOCS3, as well as SOCS1, can inhibit IFN-γ signalling. Song *et al*. showed that overexpression of SOCS3 inhibited IFN-γ-induced STAT1 phosphorylation and nuclear translocation (55). SOCS3 has also been found to inhibit IL-4-induced signalling (56). Huang *et al*. have shown that T_h1 cells have impaired IL-4 signalling. Terminally differentiated T_h1 cells showed no induction of STAT6 phosphorylation in response to IL-4, whereas STAT6 phosphorylation in IL-4-stimulated T_h2 cells was high, despite similar levels of the IL-4R expression on the surface. The authors speculate that the altered sensitivity of T_h1 cells to IL-4 may explain the stability of the T_h1 state. However, the mechanism(s) behind this impairment in IL-4 signalling are not yet known. Indeed, no SOCS1 or SOCS3 mRNA was detected in the T_h1 and T_h2 cells (17). In line with this finding, we could not detect the induction of SOCS1 mRNA in differentiated T_h2 cells in the absence of TCR re-stimulation. The requirement for TCR re-stimulation for the induction of SOCS1 in T_h2 cells suggests a possible role for SOCS1 in maintaining the phenotype of differentiated T_h2 cells. Additionally, we observed higher SOCS3 mRNA levels in T_h1 cells after TCR stimulation. However, although the level of SOCS3 induction in T_h1 is much lower than the level of SOCS1 in T_h2 cells, it is tempting to speculate, as in the case of SOCS1 in T_h2 cells, that SOCS3 in T_h1 cells suppresses possible signalling through the IL-4R. SOCS3 may be induced by IFN-γ signalling and subsequently inhibit IL-4 signalling in differentiated T_h1 cells. However, we could not find an association between the suppression of the Jak/STAT pathway and SOCS3 mRNA levels in T_h1 cells. The function of SOCS3 in T_h1 cells may be associated with non-Jak/STAT pathways, although this has yet to be investigated. Recently, Egwuagu *et al*. found a differential expression of SOCS1 in T_h1 cells and SOCS3 in T_h2 cells before and after re-stimulation with antigen (57). The contrasting results might reflect differences in the conditions under which SOCS1 and SOCS3 expression were examined, i.e. mode of T cell re-stimulation, presence of cytokines in the T_h cultures and the kinetics of SOCS expression. Furthermore, these results are also in contrast with the study by Huang *et al*., who could not detect SOCS1 and SOCS3 mRNA transcripts in non-re-stimulated T_h1 and T_h2 cells (17), which is consistent with our findings.

One of the mechanisms by which SOCS molecules inhibit cytokine signalling is through the suppression of cytokine-induced Jak activation. SOCS1 binds to Jak1, Jak2, Jak3 and Tyk2, and subsequently inhibits their activity (24,25,58,59), and SOCS3 is able to inhibit Jak2 activity following binding to activated cytokine receptors (60). However, we found no differences in the level of phosphorylation of Jak1 and Jak2 between T_h1 and T_h2 cells. Similar levels of Jak activation were seen in the different T_h subsets, despite the differences noted in SOCS mRNA levels. This might be explained if the SOCS-mediated suppression of STAT activation in T cells programmed for T_h1 and T_h2 differentiation is not dependent on the inhibition of Jak activity.

The observation that T_h1- and T_h2-specific transcription factors such as T-bet and GATA3 promote T_h1 and T_h2 differentiation in the absence of IL-12/STAT4 and IL-4/STAT6 signalling (respectively) suggests that cytokine signalling is only important in the suppression or induction of the T_h lineage commitment (15). The selective activation of STAT3 and STAT5 pathways in T_h1 cells could be an important part of the lineage selection, facilitating the selective expression of cytokines. In the light of this, it is conceivable that STAT5 is highly activated in disease-related T cells in T_h1-associated autoimmunity and is associated with the expression of T_h1 cytokines seen in these conditions.

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Abbreviations

IL-4R	IL-4 receptor
IL-12R	IL-12 receptor
Jak	Janus kinase
HRP	horseradish peroxidase
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription

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