Constitutive Activation of the Signal Transducer and Activator of Transcription Pathway in Celiac Disease Lesions

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The biological effects of interferon (IFN)- γ rely mainly on the activity of the transcription factor signal transducer and activator of transcription (STAT) 1 and the intracellular levels of suppressor of cytokine signaling (SOCS)-1, a negative regulator that controls the amplitude and duration of STAT-1 activation. IFN- γ is a key mediator of the immunopathology in celiac disease (CD, gluten-sensitive enteropathy). Thus we have investigated STAT-1 signaling and SOCS-1 expression in this condition. As expected, high local concentrations of IFN- γ were invariably seen in duodenal biopsies from CD patients in comparison to controls. On the basis of immunohistochemistry, STAT-1 phosphorylation, nuclear localization, and DNA-binding activity, STAT-1 activation was consistently more pronounced in CD compared with controls. Despite samples from CD patients containing abundant SOCS-1 mRNA, SOCS-1 protein was expressed at the same level in CD patients and controls. In explant cultures of CD biopsies, gliadin induced the activation of STAT-1 but not SOCS-1. Furthermore, inhibition of STAT-1 prevented the gliadin-mediated induction of ICAM-1 and B7-2. These data suggest that persistent STAT-1 activation can contribute to maintaining and expanding the local inflammatory response in CD. (*Am J Pathol* 2003, 162:1845–1855)

The major signal transduction pathway initiated by interferon (IFN)- γ has been elucidated by a series of elegant biochemical and genetic studies. Binding of IFN- γ to its receptor induces dimerization of the receptor chains, bringing together JAK kinases 1 and 2 that are activated by transphosphorylation. Activated JAK-1 and -2 then phosphorylate tyrosine residues within the cytoplasmic domains of the receptor subunits, which act as a docking site for a latent cytoplasmic protein, termed signal transducer and activator of transcription 1 (STAT-1). Phosphorylation of a C-terminal tyrosine (Y701) in STAT-1 facilitates interaction with the SH2 domain of a second STAT-1 molecule, mediating dimerization. STAT-1 can also be phosphorylated on a single serine residue (Ser-727) at the carboxy end of the molecule.^{1,2}

STAT-1 dimers subsequently migrate to the nucleus, where they bind to the γ -activated sequence (GAS) element contained within the promoters of IFN- γ -inducible immune-inflammatory genes (eg, IRF-1, INOS, CD86, MHC class II antigens, ICAM-1).^{1–5} Because of alternative splicing, STAT-1 exists in two forms: full-length STAT-1 α and STAT-1 β lacking 38 residues (including Ser-727) at the carboxy terminus. Only STAT-1 α is able to activate transcription of IFN- γ -responsive genes.^{1,2}

Because excessive IFN- γ stimulation might have deleterious consequences, it is not surprising that the IFN- γ /STAT-1 pathway is tightly regulated. Indeed it is known that some mechanisms are in place to modulate the cellular response to IFN- γ , and turn off the IFN- γ -activated signaling pathway.⁶ In this context, suppressor of cytokine signaling (SOCS)-1, a member of the SOCS family, plays a predominant role in suppressing IFN- γ -induced STAT-1 phosphorylation.^{7,8} SOCS-1 is an immediate early gene, and its transcripts, present at very low levels in immune cells, are rapidly up-regulated by signaling through IFN- γ /STAT-1 pathway.⁹ Thus, SOCS-1 is part of a general negative feedback loop regulating IFN- γ

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action. More recently, it has however emerged that SOCS-1 protein is unstable and that cells can contain little or no SOCS-1 protein despite high RNA level.^{10–12}

Celiac disease (CD) is an enteropathy caused by dietary gluten in genetically susceptible individuals. The characteristic features of CD inflammation are villus atrophy, crypt cell hyperplasia, and increased number of intraepithelial lymphocytes.¹³ The nature of CD pathogenesis remains unclear, but a large body of evidence indicates that CD4⁺ T cell-mediated hypersensitivity plays a major role in tissue injury in CD.¹⁴ Lamina propria CD4⁺ T cells are phenotypically activated and produce large amounts of IFN- γ when exposed to gluten.^{15,16} In addition, gliadin-specific DQ2- and DQ8-restricted IFN- γ -producing T helper (Th) 1 CD4⁺ T cells have been derived from the intestinal lamina propria of CD patients.¹⁷ These observations, together with the demonstration that direct activation of lamina propria Th1 cells in explant cultures of human fetal gut produces villous atrophy and crypt cell hyperplasia, strongly support the role of IFN- γ in the CD immunopathology.^{18–20} However, little is known about the signaling pathways and the factors that either positively or negatively regulate the IFN- γ driven biological effects in CD.

Materials and Methods

Patients and Controls

Biopsy specimens from the distal duodenum of 32 patients with untreated CD (5 to 27 years of age) were obtained during upper gastrointestinal endoscopy. The histopathological diagnosis was based on typical mucosal lesions with crypt cell hyperplasia, villous atrophy, and increased number of intraepithelial lymphocytes. All untreated CD patients were positive for anti-endomysial (EMA) and anti-gliadin (AGA) antibodies. In all untreated CD patients, at least three biopsies were collected at the time of diagnosis. One specimen was used for routine histological examination, whereas the remaining mucosal samples were immediately frozen in liquid nitrogen and stored until tested. Biopsies were also obtained from eight treated CD patients (24 to 36 years of age), who were in clinical and histological remission, and negative for AGA/EMA antibodies. No patient had gluten-refractory disease. From these patients, four or more biopsies were collected: one was used histology and the remaining for organ culture. As control, two separate groups were considered. The first group of controls comprised four patients with nonspecific duodenitis and four with food enteropathy. Duodenal biopsies taken from these patients had normal mucosal architecture with long villi and short crypts and had a mild increase in nonspecific inflammatory cells. All of the disease control patients were EMA-negative. The second group was represented by age-matched normal patients ($n = 38$) who were under investigation for gastrointestinal symptoms, but had normal histology, no increase in inflammatory cells, and were EMA- and AGA-negative. This study received

ethical approval from local committee (University Federico II, Naples, Italy).

Organ Culture and Cell Isolation

The mucosal specimens were cultured as described elsewhere.²¹ Briefly biopsies taken from patients with inactive CD were placed on iron grids with the mucosal face upwards in the central well of an organ culture dish in serum-free culture medium containing RPMI 1640 (Sigma, Milan, Italy) supplemented with 10% HL-1 (BioWhittaker, Wokingham, UK), penicillin (100 U/ml), and streptomycin (100 μ g/ml) (Life Technologies-GibcoBRL, Milan, Italy). Cultures were performed with or without the addition of 1 mg/ml of peptic-tryptic digest (Frazer III fraction) of gliadin (PT) (Sigma) in the presence or absence of the JAK/STAT inhibitor, tyrphostin B42 (TB42) (100 μ mol/L; Inalco S.P.A., Milano, Italy) or dimethyl sulfoxide (100 μ mol/L, Sigma). TB42 and dimethyl sulfoxide were preincubated for 4 hours before the addition of PT. The dishes were placed in a tight container with 95% O₂/5% CO₂ at 37°C, at 1 bar. Biopsies were snap-frozen after 24 hours and stored at –80°C until used. To examine if IFN- γ activates STAT-1 in human intestine, duodenal biopsies taken from normal controls were stimulated with rh-IFN- γ (final concentration, 100 ng/ml) (Peprotech EC Ltd., London, UK) for 1 to 4 hours, and then snap-frozen and stored at –80°C until used.

Protein Extraction and Western Blot Analysis

Western blot analysis was performed on whole mucosal duodenal samples from 12 active CD patients and 10 normal controls. Total proteins were prepared as previously described.²² For cytosolic and nuclear extracts, the method described by Schreiber and colleagues²³ was used with minor modifications. Briefly, snap-frozen biopsies were mechanically homogenized in liquid nitrogen, and cytosolic extracts collected in buffer A containing 10 mmol/L Hepes (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, and 0.2 mmol/L EGTA. Nuclear extracts were prepared by solubilizing the remaining nuclei in buffer C containing 20 mmol/L Hepes (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, and 10% glycerol. Both buffers were supplemented with 1 mmol/L dithiothreitol, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mmol/L phenylmethane sulfonyl fluoride (all reagents were from Sigma).

For the detection of IFN- γ , total proteins (100 μ g/sample) were separated on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. IFN- γ was detected using a goat anti-human IFN- γ (1:300 final dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) followed by a horseradish peroxidase-conjugated rabbit anti-goat IgG monoclonal antibody (DAKO, Cambridgeshire, UK) (final dilution, 1:2500). The reaction was detected with ECL Plus kit (Amersham Pharmaceuticals, Amersham, UK). To confirm equal loading and transfer of proteins, ponceau S (Sigma) staining was performed. In addition, after detection of IFN- γ , blots were stripped by incubation for 30 minutes at

50°C in stripping medium (2% SDS, 0.05 mol/L Tris, pH 6.8, 0.1 mmol/L β -mercaptoethanol) and analyzed for β -actin, as internal loading control, using a specific mouse anti-human β -actin antibody (1:5000 final dilution, Sigma), followed by a goat anti-mouse antibody conjugated to horseradish peroxidase (1:2500 dilution, DAKO).

To investigate STAT-1 expression, cytosolic proteins (200 μ g) were separated on a 8% SDS/PAGE gel, and analyzed for phosphorylated STAT-1 (p-STAT-1) using a mouse anti-human antibody that specifically recognizes STAT-1 phosphorylation on tyrosine 701 (1:1000 final dilution, Santa Cruz Biotechnology) or a rabbit anti-human antibody that specifically recognizes STAT-1 on serine 727 (1:5000 final dilution; Upstate Biotechnology, Lake Placid, NY). Rabbit anti-mouse or goat anti-rabbit antibody conjugated to horseradish peroxidase (1:2500 dilution, DAKO) was used as secondary antibody, and the reaction was developed with ECL-plus kit (Amersham Pharmaceuticals). After detection of p-STAT-1, blots were stripped and subsequently incubated with a rabbit anti-human STAT-1 polyclonal antibody (1:1000 final dilution, Santa Cruz Biotechnology) followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase (1:2500 dilution, DAKO).

To analyze the content of STAT-1 in the nuclear extracts, 10 μ g of nuclear protein/sample were separated on an 8% SDS/PAGE gel and analyzed using the rabbit anti-human STAT-1 polyclonal antibody as indicated above. After detection of STAT-1, blots were stripped and subsequently incubated with a mouse anti-human histone-1 monoclonal antibody (1:300 final dilution, Santa Cruz Biotechnology) followed by a goat anti-mouse antibody conjugated to horseradish peroxidase (1:1500 dilution, DAKO).

To investigate STAT-3 expression, cytosolic proteins (200 μ g) were separated on an 8% SDS/PAGE gel, and analyzed for phosphorylated STAT-3 (p-STAT-3) using a mouse anti-human antibody that specifically recognizes STAT-3 tyrosine phosphorylation (1:500 final dilution, Santa Cruz Biotechnology). Goat anti-mouse antibody conjugated to horseradish peroxidase (1:2500 dilution, DAKO) was used as secondary antibody, and the reaction was developed with ECL-plus kit (Amersham Pharmaceuticals). After detection of p-STAT-3, blots were stripped and subsequently incubated with a rabbit anti-human STAT-3 polyclonal antibody (1:1000 final dilution, Santa Cruz Biotechnology) followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase (1:2500 dilution, DAKO).

For SOCS-1 protein analysis, cytosolic proteins (100 to 200 μ g) were separated on a 12% SDS/PAGE gel and analyzed using a goat anti-human antibody that specifically recognizes SOCS-1 (1:300 final dilution, Santa Cruz Biotechnology). Rabbit anti-goat antibody conjugated to horseradish peroxidase (1:2500 dilution, DAKO) was used as secondary antibody, and the reaction was developed with ECL-plus kit (Amersham Pharmaceuticals). After detection of SOCS-1, blots were stripped and analyzed for β -actin as indicated above. SOCS-1 was also analyzed in immunoprecipitated proteins. For this purpose, 350 μ g of cytosolic proteins were immunoprecipi-

tated with an anti-SOCS-1 antibody (2 μ g/ml, Santa Cruz Biotechnology) and analyzed by Western blotting. To confirm the specificity of the SOCS-1 band, proteins extracted from the same samples were immunoprecipitated using a control isotype antibody (DAKO). A different anti-SOCS-1 antibody (1:400 final dilution, Santa Cruz Biotechnology) followed by a horseradish peroxidase-conjugated rabbit anti-goat-antibody (1:5000 dilution, DAKO) was used. Immunoreactivity was visualized using an ECL kit (Amersham Pharmaceuticals).

For SOCS-3 analysis, cytosolic proteins (100 μ g) were separated on a 12% SDS/PAGE gel and analyzed using a goat anti-human antibody that specifically recognizes SOCS-3 (1:300 final dilution, Santa Cruz Biotechnology). Rabbit anti-goat antibody conjugated to horseradish peroxidase (1:2500 dilution, DAKO) was used as secondary antibody, and the reaction was developed with ECL-plus kit (Amersham Pharmaceuticals). After detection, blots were stripped and analyzed for β -actin as indicated above.

ICAM-1 and B7-2 were examined in treated CD biopsies challenged *in vitro* with gliadin in the presence or absence of TB42. For this purpose total proteins (200 μ g/sample) were incubated with a goat anti-human B7-2 or ICAM-1 (1:300 final dilution for both, Santa Cruz Biotechnology). Rabbit anti-goat antibody conjugated to horseradish peroxidase (1:2500 dilution, DAKO) was used as secondary antibody, and the reaction was developed with ECL-plus kit (Amersham Pharmaceuticals). Bands were quantified by densitometry and values expressed as arbitrary units.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was used to detect specific binding of activated STAT-1 to GAS-containing oligonucleotides from the promoter of the human Fc γ RI gene: 5'-GTATTTCAGAAAGGAAC-3'. Nuclear protein-DNA-binding studies were performed for 20 minutes at room temperature in a 20- μ l reaction volume containing 10 mmol/L Tris, 50 mmol/L KCl, 1 mmol/L dithiothreitol, 2.5% glycerol, 5 mmol/L MgCl₂, 1 μ g poly (dl-dC), (all of the reagents were from Sigma), 50 fmol biotin-labeled GAS-containing probe, and 14 μ g of nuclear proteins. DNA probe was prepared by annealing the two consensus GAS oligonucleotides, which were labeled at the 3' end with biotin using a commercially available kit (Pierce, Rockford, IL). The binding specificity was confirmed by incubating the nuclear protein samples with unlabeled GAS probe or nonspecific oligonucleotide of the interleukin (IL)-2 gene (IL2G), 5'-CACAAACGCGTGAGCTCTCTAGAAAGCATCATCTCAACACTAAGTGAATTAAGTGCCTCGAGCA-3') in 30-fold molar excess to compete binding. In antibody blocking assay, a monoclonal mouse anti-human STAT-1 (Santa Cruz Biotechnology) or control mouse IgG antibody (DAKO) (both used at a concentration of 2.5 μ g) were incubated with the nuclear proteins for 45 minutes before adding the DNA probe. A 6% nondenaturing polyacrylamide gel was used for electrophoretic separation. After blotting to a membrane, labeled oligonucleotides were detected with a chemiluminescence EMSA kit (Pierce).

RNA Extraction, cDNA Preparation, and Southern Blotting of Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Products

RNA was extracted using 1 ml of a monophasic solution of phenol and guanidine isothiocyanate (TRIzol; Life Technologies, Paisley, UK) and chloroform, followed by isopropanol (Sigma) precipitation. The integrity of RNA was checked by electrophoresis on a 1.5% agarose gel. A constant amount of RNA (150 ng/sample) was retro-transcribed into complementary cDNA, and 1 μ l of cDNA/sample was then amplified using the following conditions: denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C for both SOCS-1 and β -actin, and 58°C for SOCS-3, and extension for 1 minute and 15 seconds at 72°C as previously reported.^{22,24}

RNA expression for SOCS-1, SOCS-3, and β -actin was then assessed semiquantitatively by Southern blotting. In preliminary experiments we established the optimal number of cycles to obtain a PCR product within the linear phase of the amplification. For this purpose an equivalent amount of cDNA for sample was amplified using specific primers for β -actin (1 μ l of cDNA for 19, 21, 23, and 25 cycles), SOCS-1 (1 μ l of cDNA for 28, 30, 31, and 33 cycles), and SOCS-3 (1 μ l of cDNA for 26, 28, 30, and 32 cycles). For Southern blot experiments cDNA samples were amplified with β -actin primers for 20 cycles, with SOCS-1 for 30 cycles, and with SOCS-3 for 26 cycles. The SOCS-1 and SOCS-3 primers were as follows: SOCS-1 forward: 5'-CACGCACTCCGCA-CATTCC-3' and SOCS-1 reverse: 5'-TCCAGCAGCTCGA-AGAGGCA-3'; SOCS-3 forward: 5'-TCACCCACAGCAA-GTTTCCCGC-3' and SOCS-3 reverse: 5'-GTTGACGGT-CTTCCGACAGAGAT-3'.

β -actin primers have been published previously.²² Parallel experiments were performed using RNA as substrate for PCR assay to exclude the amplification of genomic DNA contaminating the RNA samples. PCR product specificity was confirmed by restriction analysis. The cDNA probes used in the Southern blotting were DNA fragments encoding the full-length PCR product. RT-PCR products were run on a 1% agarose gel and Southern blotting was performed according to a commercially available chemiluminescence detection kit (Amersham International). Bands were quantified by densitometry.

Immunohistochemistry

Tissue sections were cut, deparaffinized, and dehydrated through xylene and ethanol. For the purpose of antigen retrieval, the slides were incubated in the microwave oven for 20 minutes in 0.01 mol/L of citrate buffer, pH 6 (Sigma). To block endogenous peroxidase the slides were then incubated in 2% H₂O₂ for 20 minutes at room temperature. Incubation with the human monoclonal phospho-STAT-1 antibody (Santa Cruz Biotechnology) was performed at 4°C overnight. Primary antibody, used at a final concentration of 40 μ g/ml, was omitted in sections used as negative control samples. After rinsing in Tris-buffered saline (Sigma), slides were incubated with an anti-mouse antibody conjugated to horseradish

Figure 1. A: Representative expression of IFN- γ (top) and β -actin (bottom) protein in mucosal samples taken from four patients with untreated, active, CD (celiacs) and four normal controls. The example is representative of three separate experiments analyzing in total mucosal samples from 10 patients with active CD and 9 normal controls. As positive control, recombinant human IFN- γ was used. **B:** Quantitative analysis of IFN- γ / β -actin protein ratio in mucosal samples from 10 patients with active CD (celiacs) and 9 normal controls, as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units (au). Each point represents the value (au) of IFN- γ / β -actin ratio in mucosal samples taken from a single subject. **Horizontal bars** indicate the median.

peroxidase (1:50 dilution, DAKO) for 40 minutes at room temperature. Immunoreactive cells were visualized by addition of diaminobenzidine (Sigma) as substrate and lightly counterstained with hematoxylin. Isotype control sections were prepared under identical immunohistochemical conditions, as described above, replacing the primary phospho-STAT-1 antibody with a purified, normal mouse IgG control antibody (DAKO). Tissue dehydration through graded alcohol and xylene was followed by mounting.

Results

Active STAT-1 in CD

To confirm that the CD lesion is associated with a marked expression of IFN- γ , Western blot analysis was performed using proteins extracted from duodenal mucosal samples of active CD patients and normal controls. In all CD patients and normal controls, anti-IFN- γ antibody detected a protein with a molecular size of 19 kd, co-migrating with recombinant human IFN- γ on SDS-PAGE (Figure 1). However, the intensity of the IFN- γ bands seen in CD was greater than that in normal controls (Figure 1), confirming previous reports showing that, in CD, the inflammatory response is associated with enhanced synthesis of IFN- γ .^{15,16}

Because signals through the IFN- γ receptor lead to the STAT-1 activation, we next investigated if, in CD mucosa, there was enhanced activation of STAT-1. Proteins were prepared from biopsies taken from active CD patients

Figure 2. Enhanced activation of STAT-1 in CD. **A:** Representative expression of phosphorylated STAT-1 (p-STAT-1) and total STAT-1 in mucosal samples taken from four patients with untreated CD (celiacs) and four normal controls. Cytosolic proteins, extracted from whole mucosal samples, were run on SDS-PAGE, and subsequently analyzed for p-Tyr-STAT-1 (top), p-Ser-STAT-1 (middle), and total STAT-1 (bottom) as indicated in Materials and Methods. The example is representative of three separate experiments analyzing in total mucosal samples from 10 patients with CD and 9 normal controls. +ve = THP-1 cells stimulated with rh-IFN- γ (30 ng/ml) for 30 minutes. **B:** Representative blot of STAT-1 in nuclear proteins extracted from four patients with untreated CD (celiacs) and four normal controls. Nuclear proteins, extracted from whole mucosal samples, were run on SDS-PAGE, and subsequently analyzed for STAT-1 (top) and histone-1 (bottom) as indicated in Materials and Methods. The example is representative of three separate experiments analyzing in total mucosal samples from eight patients with CD and eight normal controls. +ve = THP-1 cells stimulated with rh-IFN- γ (30 ng/ml) for 30 minutes.

and normal controls and separated by SDS-PAGE. Phosphorylation of STAT-1 (p-STAT-1) was then monitored by Western blotting analysis using an antibody that specifically recognize STAT-1 phosphorylation on tyrosine 701 (p-Tyr-STAT-1). As shown in Figure 2A (top blot), a strong band corresponding to p-Tyr-STAT-1 α was seen in CD. In contrast, STAT-1 was weakly phosphorylated on the tyrosine 701 residue in normal controls (Figure 2A, top blot) and patients with duodenitis or food enteropathy (not shown). Although STAT-1 tyrosine phosphorylation is essential and sufficient to allow formation of DNA-binding STAT dimers, STAT-1 dimers will be transcriptionally inactive unless phosphorylated on serine 727.^{25,26} Therefore we then used an antibody, which specifically recognizes STAT-1 molecules phosphorylated on serine 727 (p-Ser-STAT-1) to monitor the phosphorylation of this critical STAT-1 residue. The data in Figure 2A, middle blot, show that mucosal samples taken from active CD patients exhibit a more pronounced p-Ser-STAT-1 α in comparison to normal controls. Reprobing the blots with a pan-STAT-1 antibody demonstrated that approximately equal amounts of STAT-1 α and STAT-1 β were present in all of the samples (Figure 2A, bottom blot).

After phosphorylation, STAT-1 rapidly translocates to the nucleus where it binds to specific DNA sequences. Consistent with this, we found that immunoreactivity for STAT-1 was greater in nuclear extracts of duodenal biopsies from active CD patients than in normal controls (Figure 2B). Analysis of STAT-1 α /histone-1 ratios showed

Figure 3. **A:** Representative EMSA blot showing enhanced STAT-1-DNA-binding activity in CD. Nuclear proteins extracted from mucosal biopsies from three patients with CD (celiacs) and three normal controls were analyzed as indicated in Materials and Methods. Unstimulated and IFN- γ -stimulated THP-1 cells were used as negative and positive controls, respectively. In the first lane, no nuclear extract was run. STAT-1-binding GAS complex is retarded, whereas unbound (free) DNA probes migrate to the bottom of the electropherogram. One of three separate experiments analyzing samples from eight patients with CD and eight controls is shown. **B:** Representative EMSA blot showing the specificity of the STAT-1-binding complex. Incubation of nuclear proteins, extracted from a mucosal biopsy of a patient with CD, with a monoclonal STAT-1 antibody or excess of specific GAS probe, but not with a nonrelevant control antibody or excess of nonspecific DNA, results in a complete disappearance of the STAT-1-binding DNA complex. One of three representative experiments is shown.

a significantly higher value in patients with active CD (median, 0.84 densitometry arbitrary units; range, 0.6 to 1.11 arbitrary units) than in normal controls (median, 0.04 arbitrary units; range, 0.01 to 0.076 arbitrary units) ($P < 0.01$). Additionally, EMSA analysis demonstrated higher levels of specific oligonucleotide binding activity of STAT-1 in active CD than in normal controls (Figure 3).

To show that local increased IFN- γ production can enhance STAT-1 activation, we cultured duodenal biopsies taken from normal controls with rh-IFN- γ and then assessed STAT-1 activation. As shown in Figure 4, stimulation of normal duodenal biopsies with IFN- γ results in enhanced STAT-1 activation, which peaks at 1 hour and returns at basal level after 4 hours of culture.

Lamina Propria Mononuclear Cells Express High Levels of STAT-1 in CD

To localize the anatomical site of cells that contain STAT-1, we performed immunohistochemical analysis of both CD and normal duodenal sections using a p-Tyr-STAT-1 antibody. Virtually all of the lamina propria mononuclear cells (LPMCs) exhibit a nuclear accumulation of p-STAT-1 in CD, whereas considerably less p-STAT-1-positive LPMCs were seen in normal duodenal mucosa (Figure 5, bottom left). In addition, p-STAT-1 staining in LPMCs was more intense in CD than in normal controls. Finally, p-STAT-1 also localized within the nucleus of epi-

Figure 4. IFN- γ enhances STAT-1 activation in normal duodenum. **A:** Representative expression of phosphorylated STAT-1 on tyrosine residues (p-Tyr-STAT-1) and total STAT-1 in duodenal mucosal samples taken from normal controls, and cultured in medium alone (UNST) or in the presence of IFN- γ (100 ng/ml) for 1, 2, or 4 hours. Cytosolic proteins, extracted from whole mucosal samples, were run on SDS-PAGE, and subsequently analyzed for p-Tyr-STAT-1 (**top**) and total STAT-1 (**bottom**) as indicated in Materials and Methods. The example is representative of three separate experiments analyzing in total mucosal samples from three normal controls. **B:** Representative blot of STAT-1 (**top**) and histone-1 (**bottom**) in nuclear proteins extracted from duodenal mucosal samples taken from normal controls and cultured in the presence or absence of IFN- γ (100 ng/ml) for 1, 2, or 4 hours. Nuclear proteins, extracted from whole mucosal samples, were run on SDS-PAGE, and subsequently analyzed for STAT-1 or histone-1 as indicated in Materials and Methods. The example is representative of three separate experiments analyzing in total mucosal samples from three normal controls.

thelial cells in both CD and normal controls. The specificity of these findings was confirmed using a nonrelevant isotype control antibody (Figure 5, top and bottom right panels).

Expression of SOCS-1, an Inhibitor of STAT-1, in CD Mucosa

In response to IFN- γ challenge, activation of STAT-1 pathway eventually leads to the induction of SOCS-1, a protein that inhibits up-stream kinases and turns off the IFN- γ /STAT-1 signal transduction pathway.^{7,9} Recent reports have demonstrated that SOCS-1 is regulated also at posttranscription level and that protein expression can be repressed through various mechanisms.^{10–12} We first analyzed the content of SOCS-1 RNA by Southern blotting of RT-PCR products. Mucosal samples taken from active CD patients contained higher levels of SOCS-1 than normal controls (Figure 6A). SOCS-1 protein was then analyzed in all of the mucosal samples assessed for p-STAT-1 by Western blotting. However using two different commercially available antibodies we were unable to detect SOCS-1 protein, regardless of whether biopsies taken from treated or untreated CD or normal controls (Figure 6B). Importantly, the same antibodies were able to detect SOCS-1 protein in IFN- γ -stimulated THP-1 cells (Figure 6B). These data suggest that in duodenal biopsies, SOCS-1 protein concentrations are below the de-

tection limit of Western blotting. We then used immunoprecipitation followed by Western blotting to improve the sensitivity of detection. By this technique SOCS-1 protein was detected in CD patients and normal controls (Figure 6C), however no difference was seen. Furthermore, no SOCS-1 protein was seen in both CD and normal controls by immunohistochemistry (data not shown).

Enhanced STAT-3 Activation Is Associated with High SOCS-3 Expression in CD Mucosa

Binding of IFNs to their receptors induces activation of STAT-3 other than STAT-1. We have recently shown that in untreated CD there is enhanced production of IFN- α , and that stimulation of lamina propria T cells in explants of human fetal gut with anti-CD3 and IFN- α results in activation of both STAT-1 and STAT-3.^{27,28} Therefore, we then assessed if in CD mucosa there is enhanced activation of STAT-3. As shown in Figure 7A, STAT-3 was strongly phosphorylated in CD mucosal samples. Moreover, we were able to show that expression of SOCS-3, a downstream gene in the STAT-3 signaling cascade, was more pronounced in CD than in controls. This was evident at both the RNA and protein level (Figure 7, B and C).

In Treated CD Biopsies, Inhibition of STAT-1 Prevents the Gliadin-Mediated Induction of Co-Stimulatory Molecules

The enhanced STAT-1 activation seen in CD prompted us to explore the functional role of this transcription factor in the inflammatory response in CD. To address this issue, we used an established *ex vivo* organ culture of treated CD biopsies.²¹ First we showed that in treated CD biopsies, 24-hour stimulation with gliadin resulted in enhanced phosphorylation of STAT-1 α on both tyrosine and serine residues (Figure 8A) with no induction in SOCS-1 protein (Figure 8B). Immunoprecipitated proteins from unstimulated and gliadin-treated biopsies exhibited similar levels of SOCS-1 (Figure 8C). Because ICAM-1 and B7-2, two co-stimulatory molecules involved in the pathogenesis of CD, are mostly regulated by STAT-1,^{4,29–31} we then assessed if inhibition of STAT-1 in CD biopsies prevented the gliadin-mediated induction of both these co-stimulatory molecules. As expected, stimulation of CD biopsies with gliadin enhanced both ICAM-1 and B7-2 expression (Figure 9). The addition of TB42, a JAK-2/STAT-1 pathway inhibitor, in the organ culture prevented the gliadin-induced STAT-1 activation and this was associated with a dramatic decrease in both ICAM-1 and B7-2 (Figure 9).

Discussion

In this study we provide evidence that activation of STAT-1 is an important feature of the inflammatory response in CD. Using different assays, we show that all of the critical steps in the STAT-1 signal transduction pathway (ie, phosphorylation of cytoplasmic protein, translo-

Figure 5. Immunohistochemical staining for phosphorylated STAT-1 (p-STAT-1) in paraffin-embedded sections from CD and normal control intestinal tissue sections. Using a phosphotyrosine STAT-1 monoclonal antibody, duodenal mucosa of a patient with CD reveals a nuclear accumulation of p-STAT-1 in epithelial cells and virtually all of the LPMCs (**top left**). In the duodenal mucosa of a normal control, p-STAT-1 also localizes within the nucleus of epithelial cells as well as LPMCs (**bottom left**). However, p-STAT-1 staining, especially in LPMCs, is less diffuse and intense than that observed in CD. In the **right** panels (both **top** and **bottom**), control immunohistochemical reactions using an isotype mouse IgG that correspond to the sections in the **left** panels (**top** and **bottom**), are shown. The example is representative of six separate experiments, in which biopsies taken from six patients with active CD and six normal controls were analyzed. Original magnifications, $\times 40$.

cation to the nucleus, and binding to specific DNA sequences) are enhanced in CD in comparison to normal controls. By immunohistochemistry, STAT-1 is seen within the nucleus of epithelial and virtually all of the lamina propria mononuclear cells of CD patients, whereas in normal controls immunostaining is less diffuse and intense. In addition we demonstrate that stimulation of treated CD biopsies with gliadin is followed by the induction of functionally active STAT-1. Altogether, our data are consistent with recent studies showing enhanced activation of STAT-1 in other human inflammatory diseases.^{32,33}

The exact mechanism that underlies the sustained STAT-1 activation in CD remains uncertain, but several possibilities can be pointed out. First, it is reasonable to presume that the overproduction of IFN- γ plays a major role. Indeed, STAT-1 is mostly activated in response to IFN- γ ,¹ and in this study we showed that, in CD mucosal samples, exaggerated IFN- γ was invariably associated with high STAT-1 activation, and that stimulation of normal duodenal biopsies with IFN- γ enhanced STAT-1 activation. In addition to IFN- γ , other cytokines (eg, IFN- α , IL-2, IL-6, IL-10) and a variety of growth factors can

Figure 6. SOCS-1 in CD. **A:** Southern blot analysis of transcripts for SOCS-1 and β -actin in duodenal mucosal tissue homogenates from four patients with untreated CD (celiacs) and four normal controls. cDNA samples were amplified using specific primers for SOCS-1 or β -actin, respectively. RT-PCR products were separated on agarose gel, blotted, and hybridized with specific probes for SOCS-1 or β -actin. The example is representative of three separate experiments analyzing in total mucosal samples from 10 patients with CD and 10 controls. **B:** Western blot analysis of SOCS-1 and β -actin protein expression in mucosal samples taken from the distal duodenum of four patients with CD and four normal controls. +ve = THP-1 cells stimulated with rh-IFN- γ (30 ng/ml) for 24 hours. After stripping, the blot was incubated with an anti- β -actin Ab as internal loading control. One of two representative experiments analyzing in total mucosal samples from 10 patients with CD and 9 normal controls is shown. **C:** Western blot analysis of SOCS-1 in immunoprecipitated protein samples taken from the distal duodenum of four patients with CD (lanes 1 to 4) and four normal controls (lanes 6 to 9). In lane 5, proteins extracted from a CD biopsy (the same shown in lane 4) were immunoprecipitated with a control goat IgG and then incubated with the SOCS-1 antibody. One of two representative experiments analyzing in total mucosal samples from six patients with CD and six normal controls is shown. In both experiments, a control isotype antibody was used to confirm the specificity of the immunoprecipitated band.

however activate STAT-1.¹ Independent of the system used however activation of STAT-1 is a transitory event, which peaks at 30 to 120 minutes after stimulation and then rapidly disappears. It has recently emerged that a protein belonging to the SOCS family, ie, SOCS-1, is able to prevent STAT-1 phosphorylation by inhibiting upstream kinases. Indeed, enforced expression of SOCS-1 in several cell lines represses IFN- γ -induced STAT-1 function.⁶⁻⁹ Furthermore mice lacking SOCS-1 exhibit monocytic and polymorphonuclear infiltration of several organs, fatty degeneration of the liver, and early neonatal lethality.^{7,8} These severe pathological changes are because of exaggerated IFN- γ -mediated STAT-1 activity, because IFN- γ or STAT-1 deficiency protects SOCS-1 knockout mice from disease.^{7,34,35} Importantly, SOCS-1 is induced by the IFN- γ /STAT-1 pathway, and therefore participates in a negative feedback loop.⁹ In agreement with this, we found an enhanced content of RNA transcripts for SOCS-1 in both CD mucosal samples contain-

Figure 7. **A:** Representative expression of phosphorylated STAT-3 (p-STAT-3) and total STAT-3 in mucosal samples taken from three patients with untreated CD (celiacs) and three normal controls. Cytosolic proteins, extracted from whole mucosal samples, were run on SDS-PAGE, and subsequently analyzed for p-Tyr-STAT-3 (top) and total STAT-3 (bottom) as indicated in Materials and Methods. The example is representative of two separate experiments analyzing in total mucosal samples from seven patients with CD and seven normal controls. **B:** Southern blot analysis of transcripts for SOCS-3 and β -actin in duodenal mucosal tissue homogenates from three patients with untreated CD (celiacs) and three normal controls. cDNA samples were amplified using specific primers for SOCS-3 or β -actin, respectively. RT-PCR products were separated on agarose gel, blotted, and hybridized with specific probes for SOCS-3 or β -actin. The example is representative of three separate experiments analyzing in total mucosal samples from eight patients with CD and seven normal controls. **C:** Western blot analysis of SOCS-3 and β -actin protein expression in mucosal samples taken from the distal duodenum of three patients with CD and three normal controls. +ve = THP-1 cells stimulated with rh-IFN- γ (30 ng/ml) for 24 hours. After stripping, the blot was incubated with an anti- β -actin Ab as internal loading control. One of two representative experiments analyzing in total mucosal samples from nine patients with CD and eight normal controls is shown.

ing high levels of active STAT-1. However, no SOCS-1 protein was seen in CD and normal controls by Western blotting and immunohistochemistry. This observation would seem to be a surprise given the high level of SOCS-1 RNA seen in CD mucosa. It is however noteworthy that our data are consistent with those from recent studies that failed to detect SOCS-1 protein in cells containing high levels of SOCS-1 RNA.^{5,11,36} The most plausible explanation for our results is that, in the human mucosal gut, SOCS-1 protein concentration is below the detection limit of Western blotting. Indeed, SOCS-1 was detected after immunoprecipitating high amount of proteins. No difference in SOCS-1 protein was however seen between CD patients and normal controls. This raises the possibility that, in CD, SOCS-1 protein is rapidly degraded after its synthesis, consistently with the demon-

Figure 8. A: Stimulation of treated CD biopsies with gliadin (PT) results in enhanced STAT-1 activation. Cytosolic proteins were run on SDS-PAGE, and subsequently analyzed for p-Tyr-STAT-1 (top), p-Ser-STAT-1 (middle), and total STAT-1 (bottom) as indicated in Materials and Methods. Two of six experiments analyzing in total mucosal samples from six patients with treated CD stimulated with gliadin are shown. +ve = THP-1 cells stimulated with rh-IFN- γ (30 ng/ml) for 30 minutes. B: Western blot analysis of SOCS-1 and β -actin protein expression in mucosal samples taken from patients with treated CD and stimulated *in vitro* with gliadin for 24 hours. Two representative experiments analyzing in total mucosal samples from five patients with treated CD stimulated with gliadin are shown. +ve = THP-1 cells stimulated with rh-IFN- γ (30 ng/ml) for 24 hours. Inset: Southern blot analysis of transcripts for SOCS-1 and β -actin in mucosal samples taken from patients with treated CD and stimulated *in vitro* with gliadin for 24 hours. Two representative experiments analyzing in total mucosal samples from four patients with treated CD stimulated with gliadin are shown. C: Western blot analysis of SOCS-1 in immunoprecipitated protein samples taken from patients with treated CD and stimulated *in vitro* with gliadin for 24 hours. One of two representative experiments analyzing in total mucosal samples from four patients with CD is shown. In both experiments, a control isotype antibody was used to confirm the specificity of the immunoprecipitated band.

stration that the highly conserved C-terminal homology domain, termed SOCS box, can interact with elongins B and C, thereby coupling SOCS proteins to the proteasome protein degradation pathway.¹⁰

Immunostaining revealed nuclear STAT-1 in virtually all cells in active CD, which is not surprising considering the local abundance of IFN- γ . However, there is the possibility that SOCS-1 protein is present in low concentration in different cell types, although we could not visualize this by standard immunostaining. We think that is important to examine STAT-1 and SOCS-1 in the different cell types in inflamed mucosa because there will almost certainly be cell-specific regulation of the downstream effect of STAT-1 activation. The barrier to progress in this area is technical in that it is not possible with the present technologies to extract sufficient proteins from either purified T cells, epithelial cells, macrophages, or fibroblasts from small biopsies to perform Western blotting.

In the gut there is a delicate balance between the need to recognize antigens of pathogenic microorganisms with the need to prevent unwanted immune responses to foods or the normal flora. The site at which this occurs is in the organized lymphoid tissue of the Peyer's patches.³⁷ We have recently demonstrated that T cells in human Peyer's patches respond to dietary proteins releasing high levels of IFN- γ , because of the local production of IL-12.^{38,39} After sensitization in the Peyer's patches, T cells leave via the lymphatics, enter the blood, and mi-

grate back to the lamina propria, where they rapidly undergo apoptosis.³⁷ In addition T cells in the lamina propria are actively down-regulated by immunosuppressive molecules, such as IL-10, transforming growth factor- β , and prostaglandin E2.³⁷ These mechanisms thus prevent the development of intestinal disease by limiting Th1 cell activity. In contrast, in CD, sensitized T cells arriving from the Peyer's patches may recognize low amounts of dietary gluten presented on lamina propria APC and be triggered because of the enhanced expression of co-stimulatory molecules. In this context, activation of STAT-1 can be crucial, because there is wide evidence that it regulates the synthesis of co-stimulatory molecules.^{1,2,5,31} Consistent with this, we here show that inhibition of STAT-1 activation prevents the gliadin-mediated induction of ICAM-1 and B7-2 in CD biopsies. By enhancing ICAM-1, STAT-1 could also facilitate recruitment of inflammatory cells into the intestinal mucosa. Another potential contributor to the immune-inflammatory response in CD is related to the ability of STAT-1 to regulate the differentiation of Th1 cells. This relies in part on the induction of IRF-1, a transactivator required for the synthesis of Th1-inducing cytokines.^{40,41} Importantly, in CD mucosa, STAT-1 activity corresponds closely with increased IRF-1 expression (Salvati V et al, manuscript submitted for publication). Furthermore the diminished production of IFN- γ by T cells from STAT-1-deficient mice⁴² and the increased level of IFN- γ in circulation in mice deficient for SOCS-1⁸ suggest that STAT-1 can directly influence IFN- γ expression. Indeed, several STAT-binding sites have been identified within an enhancer located at the first intron of the IFN- γ gene and STAT-1 binds to four of them. Additional STAT-1 binding sites have been found in the promoter region of the IFN- γ gene.⁴³

Figure 9. Inhibition of STAT-1 activation prevents the gliadin-mediated induction of ICAM-1 and B7-2. Treated CD biopsies were preincubated with tyrphostin B42 (TB42), a JAK-2-STAT-1 inhibitor, or dimethyl sulfoxide for 4 hours before the stimulation with gliadin (PT) for further 20 hours. STAT-1 [both tyrosine-phosphorylated (p-Tyr) and total forms], ICAM-1, and B7-2 expression were examined by Western blotting as indicated in Materials and Methods. One of three experiments analyzing in total mucosal samples from three patients with treated CD stimulated with gliadin are shown. M = biopsies cultured with only medium.

Phosphorylated STAT-1 was also seen in all duodenal mucosal samples from normal controls, and nuclear protein preparations from the same samples exhibited STAT-1-binding DNA activity. We would however like to emphasize that this finding is not surprising, given that all normal duodenal mucosal samples also contain IFN- γ , the major inducer of STAT-1 activation.

Consistent with our previous data showing up-regulation of IFN- α in CD mucosa and the ability of IFN- α to promote STAT-3 activation in human gut,^{27,28} we show that STAT-3 phosphorylation is enhanced in CD mucosa. We also provide evidence that in CD mucosa there is exaggerated expression of SOCS-3, a downstream gene in the STAT-3 signaling pathway.⁶ Importantly, up-regulation of SOCS-3 occurs at both the RNA and protein level, clearly indicating that, in CD mucosa, there is a different regulation in the expression of SOCS-1 and SOCS-3 protein. As SOCS-3 plays a crucial role in limiting STAT-3 phosphorylation, we however feel that the amount of SOCS-3 produced in CD mucosa is not sufficient to suppress STAT-3 activation.⁶

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