Cutting Edge: Silencing Suppressor of Cytokine Signaling 3 Expression in Dendritic Cells Turns CD28-Ig from Immune Adjuvant to Suppressant

Ciriana Orabona, Maria Laura Belladonna, Carmine Vacca, Roberta Bianchi, Francesca Fallarino, Claudia Volpi, Stefania Gizzi, Maria Cristina Fioretti, Ursula Grohmann and Paolo Puccetti

*J Immunol* 2005; 174:6582-6586;
doi: 10.4049/jimmunol.174.11.6582
http://www.jimmunol.org/content/174/11/6582

**References**
This article *cites 24 articles*, 13 of which you can access for free at:
http://www.jimmunol.org/content/174/11/6582.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Silencing Suppressor of Cytokine Signaling 3 Expression in Dendritic Cells Turns CD28-Ig from Immune Adjuvant to Suppressant

Ciriana Orabona, Maria Laura Belladonna, Carmine Vacca, Roberta Bianchi, Francesca Fallarino, Claudia Volpi, Stefania Gizzi, Maria Cristina Fioretti, Ursula Grohmann, and Paolo Puccetti

CTLA-4-Ig and CD28-Ig are both agonist ligands of B7 coreceptor molecules on mouse dendritic cells (DCs), yet they bias the downstream response in opposite directions, and CTLA-4-Ig promotes tolerance, whereas CD28-Ig favors the onset of immunity. Although B7 engagement by either ligand leads to a mixed cytokine response, a dominant IL-6 production in response to CD28-Ig prevents the IFN-γ-driven induction of immunosuppressive tryptophan catabolism mediated by IDO. In the present study, we show that silencing the expression of suppressor of cytokine signaling 3 (SOCS3) in DCs by RNA interference renders CD28-Ig capable of activating IDO, likely as a result of unrestrained IFN-γ signaling and IFN-γ-like actions of IL-6. Thus, in the absence of SOCS3, CD28-Ig becomes immunosuppressive and mimics the action of CTLA-4-Ig on tryptophan catabolism. The Journal of Immunology, 2005, 174: 6582–6586.

Suppressor of cytokine signaling (SOCS) proteins have emerged as critical modulators of cytokine-mediated processes (1). Not only does the feedback inhibitor SOCS3 attenuate IL-6 signaling (2) but up-regulation of SOCS3 by IL-6 may be responsible for inhibition of STARDependent signaling by IFN-γ (3). In addition to modulation of IFN-γ-inducible genes, a series of recent studies with various experimental approaches have demonstrated that SOCS3 is involved in the prevention of IFN-γ-like responses in hepatocytes and macrophages stimulated with IL-6 (4–6). It has been suggested that, in the absence of SOCS3, IL-6 may become immunosuppressive, activating genes typically induced by IFNs (7).

In murine dendritic cells (DCs), SOCS3 plays a critical role in orchestrating immune responses (8). SOCS3 attenuates IL-6 signaling (2) but up-regulates SOCS3 and prevents IDO activation by IL-6. SOCS3 silencing therefore renders CD28-Ig capable of activating IDO, likely as a result of unrestrained IFN-γ signaling and IFN-γ-like actions of IL-6. Thus, in the absence of SOCS3, CD28-Ig becomes immunosuppressive and mimics the action of CTLA-4-Ig on tryptophan catabolism.

Materials and Methods

Mice and reagents

Female DBA/2J (H-2b) mice were obtained from Charles River Breeding Laboratories (Calco). Neutralizing XMG1.2 mAb to IFN-γ and mAbs 6B4 (anti-mouse IL-6) and 15A7 (anti-mouse IL-6R) were described previously (11). CTLA-4-Ig and CD28-Ig were fusion proteins generated from the extracellular domains of murine CTLA-4 and CD28, respectively, and the Fc portion of a murine IgG3, with native IgG3, or the Fc portion of IgG3 alone (Ig-C). The enzyme inhibitor 1-methyl-DL-tryptophan (1-MT) was purchased from Sigma-Aldrich (Milan, Italy). The P815AB (amino acid sequence LPYLGWLVF) peptide was synthesized and purified as described previously (14, 15). All in vivo studies were done in compliance with National and Perugia University Animal Care and Use Committee guidelines.

DC preparations and treatments and immunization

Splenic DCs were purified by MACS using CD11c MicroBeads and MidiMacs (Miltenyi Biotec) in the presence of EDTA to disrupt DC-T cell complexes (14, 15). Cells were >99% CD11c+ (>99% MHC I-A+, >98% B7-2, <0.1% CD3+, and appeared to consist of 90–95% CD8+, 5–10% CD8−, and 1–5% B220+ cells. Additionally, DC populations were separated into CD8− and CD8+ fractions by means of CD8α MicroBeads (Miltenyi Biotec) (14, 15). The CD8− fraction was ~45% CD4+ and typically contained <0.5% containing CD8− cells. Less than 1% CD8− and <5% CD8− DCs expressed...
the B220 marker, respectively (16). DCs were exposed to 40 μg/ml CTLA-4-Ig, CD28-Ig, IgG3, or Ig-Cys or to 20 ng/ml IL-6 for 24 h at 37°C in the presence or absence of 2 μM 1-MT (11, 14). For cytoreduction, DCs were exposed to fusion proteins in vitro in the presence of 6B4 and 15A7 mAbs (for IL-6 neutralization; each at 10 μg/ml) or anti-mouse IFN-γ mAbs (10 μg/ml) as described previously (11). For immunization, cells were washed between and after incubations before peptide loading (5 μM, 2 h at 37°C), irradiation, and i.v. injection into recipient hosts. A total of 3 × 10^7 CD8+ (or, where indicated, CD8-ig-treated CD8+) DCs was injected either alone or in combination with 3% CD8+ DCs.

**siRNA synthesis and transfection**

The siRNA sequences specific for murine SOCS3 (sense, 5′-GGAGGCAAAAGGGUCAGAGG-3′; antisense, 5′-CCUCUGACCCCUUUGCUCCtt-3′) were selected, synthesized, and annealed by the manufacturer (Ambion). For transfection, siRNAs (6.7 μg) in 30 μl of transfection buffer (20 mM HEPES and 150 mM NaCl (pH 7.4)) were pipetted into a sterile Eppendorf tube. In a separate polystyrene tube, 6.7 μg of 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) were mixed with 30 μl of transfection buffer, and then both solutions were mixed gently by pipetting several times. After incubation at room temperature for 20 min, the mixture was added to 1 ml of complete medium containing 10^6 DCs and incubated for 24 h at 37°C in the presence or absence of IL-6 or CD28-Ig. Cells were then recovered, washed, and immediately used for in vitro or in vivo experiments. siRNA treatment resulted in complete disappearance of SOCS3 transcripts at 24–48 h. Control treatments consisted of either or both of the different controls: mock-transfected DCs, i.e., cells treated with DOTAP alone (17), and Negative Control siRNA (Ambion). Expression of SOCS3 (sense, 5′-GCGATGGTCCACCCAGGAACCATT-3′; antisense, 5′-AAGGTGGCATCATCTATCGGAG-3′) and control SOCS1 (sense, 5′-TGTAGGACCAAGCACAGG-3′; antisense, 5′-GGAGGAGGAGGAGGAGGAGG-3′) transcripts was evaluated by RT-PCR analysis using specific primers.

**Skin test assay**

A skin test assay was used for measuring class I-restricted delayed-type hypersensitivity responses to synthetic peptides as described previously (11, 17). Results were expressed as the increase in footpad weight of peptide-injected footpads over that of vehicle-injected counterparts. Data are the mean ± SD for at least six mice per group. The statistical analysis was performed using Student’s paired t test by comparing the mean weight of experimental footpads with that of control counterparts. The data reported are representative of at least three independent experiments.

**Kynurenine assay**

IDO functional activity was measured in vitro in terms of the ability of DCs to metabolize tryptophan to kynurenine, the concentrations of which were measured by HPLC as described previously (8).

**Western blot analysis**

IDO expression was investigated as previously described using a specific Ab (9). On studying STAT phosphorylation, CD8+ DCs were exposed to IL-6 (20 ng/ml) for 10 min. After SDS-PAGE resolution, immunoblotting was performed by sequential exposure to anti-phospho-STAT1/anti-STAT1 or anti-phospho-STAT3/anti-STAT3 Abs, as described previously (17, 18).

**Results and Discussion**

**CTLA-4-Ig and CD28-Ig have disparate effects on Ag presentation by DCs**

DC populations in the spleens of DBA/2 mice consist of CD8+ (~90%) and CD8- (~10%) fractions that mediate the respective immunogenic and tolerogenic presentation of the synthetic tumor/self nonapeptide P815AB (19). Upon transfer into recipient hosts, peptide-loaded CD8+ DCs initiate immunity, and CD8- DCs initiate anergy when Ag-specific skin test reactivity is measured at 2 wk after cell transfer (14, 20). In line with previous results of functional plasticity of DC subsets (9, 15), Fig 1A shows that otherwise immunogenic CD8+ DCs became tolerogenic as a result of DC exposure to CTLA-4-Ig before peptide loading and transfer into recipient mice. The effect of CTLA-4-Ig on CD8+ DCs was contingent upon autocrine IFN-γ and the expression of functional IDO because it was negated by the presence of either IFN-γ-neutralizing Ab or the enzyme inhibitor 1-MT during DC exposure to CTLA-4-Ig. Consistent with previous results of adjuvant activity by CD28-Ig (11), otherwise tolerogenic CD8+ DCs were made capable of immunogenic presentation of P815AB when exposed to CD28-Ig before peptide loading and transfer into recipient hosts, which was dependent on autocrine IL-6 (Fig 1B). Thus, CTLA-4-Ig and CD28-Ig affect the functional plasticity of DC subsets in opposite directions through the respective dominant actions of autocrine IFN-γ and IL-6. As in other model systems with CTLA-4-Ig (8, 16, 21), tolerogenesis by CTLA-4-Ig acting on CD8- DCs requires tryptophan catabolism.

**SOCS3 is required for the effect of CD28-Ig and IL-6 on CD8+ DCs**

The default tolerogenic potential of CD8+ DCs is such that as few as 3% CD8+ DC admixed with CD8- DCs will negate the induction of immunity to P815AB by the latter cells upon transfer into recipient hosts (22, 23). However, the effect is no longer observed when the CD8+ DC fraction is treated with rIL-6 before peptide loading and mixing with CD8- DCs (14). We examined whether SOCS3 expression is required for CD28-Ig and IL-6 effects on CD8+ DCs. Splenic DCs were fractionated, and specific SOCS3 gene silencing was achieved in the CD8+ fraction with siRNA technology (Fig 2A). P815AB-pulsed CD8- DCs were injected either alone or in combination with 3% CD8+ DCs, either untreated or treated with CD28-Ig.
or rIL-6, with or without concomitant siRNA-SOCS3 treatment (Fig. 2B). The results showed that SOCS3 was an absolute requirement for the occurrence of regulatory effects by CD28-Ig and rIL-6 on CD8+ DCs. Thus, in the absence of SOCS3, CD8+ DCs are refractory to immunomodulation by endogenous or externally added IL-6, leaving their default tolerogenic program apparently unaffected. This is consistent with the view that IL-6 cannot down-regulate the expression of IFN-inducible genes, and possibly IDO, in cells lacking SOCS3 (3).

CD28-Ig treatment equates to CTLA-4-Ig treatment in CD8+ DCs lacking SOCS3

Apart from the effects of IL-6 on CD8+ DCs, the cytokine can directly reinforce the basal immunogenic activity of CD8+ DCs when these cells are treated with rIL-6 and then admixed with untreated CD8+ DCs, thus enabling the development of P815AB-specific skin test reactivity (14). However, this effect is lost when the CD8+ DC fraction is treated with CTLA-4-Ig before peptide loading and mixing with CD8+ DCs (15). We compared the effects of CTLA-4-Ig and CD28-Ig on untreated and siRNA-SOCS3-treated CD8+ DCs, respectively, when these cells were cotransferred with CD8- DCs activated by IL-6. We assayed the development of P815AB-specific skin test reactivity in hosts transferred with IL-6-treated CD8- DCs, either alone or in combination with 3% cells of the other subset, treated with CTLA-4-Ig, CD28-Ig or rIL-6, with or without SOCS3 siRNA (Fig. 3). In line with previous results, CTLA-4-Ig-treated CD8+ DCs were found to inhibit priming by IL-6-conditioned cells of the other subset (9, 15). Remarkably, the same pattern of reactivity was displayed by CD8+ DCs exposed to CD28-Ig or IL-6 in the presence of SOCS3 siRNA. Also, the effect of CTLA-4-Ig on CD8+ DCs as well as that of CD28-Ig on SOCS3-deficient CD8+ cells was due to active suppression involving IDO because it could be reversed by the addition of the enzyme inhibitor 1-MT during cell exposure to CD28-Ig (Fig. 3). Thus, the functional effects of CD28-Ig on CD8+ DCs are remarkably similar to those of CTLA-4-Ig once SOCS3 expression has been silenced. It has been shown that IL-6 strongly activates STAT3 and induces the expression of IFN-responsive genes in SOCS3-deficient macrophages, indicating that IL-6 might mimic the actions of IFNs (4, 5). Although it is possible that lack of SOCS3 enables efficient IFN-γ signaling in DCs treated with CD28-Ig, IL-6 might also induce IFN-γ-like responses, including activation of IDO, in DCs lacking SOCS3.

IL-6 induces STAT3 phosphorylation, IDO expression, and tryptophan catabolism in siRNA-SOCS3-treated CD8+ DCs

To ascertain whether IL-6 will induce an IFN-γ-like response through STAT proteins in DCs lacking SOCS3, we assayed the cytokine for possible direct effects on the induction of immunosuppressive tryptophan catabolism. STAT1 phosphorylation is normally required for IFN-γ-induced transcription of the Indole gene (8, 17, 18). However, prolonged STAT3 activation is observed in response to IL-6 in cells lacking SOCS3, and STAT3 may be involved in the activation of IFN-inducible genes by IL-6 (7). We measured STAT1 and STAT3 phosphorylation in response to IL-6, IDO expression by immunoblot analysis, and tryptophan conversion to kynurenine in siRNA-SOCS3-treated and control CD8+ DCs. Limited STAT1 phosphorylation was observed in response to IL-6 in control cells, and the extent of phosphorylation was not enhanced by the absence of SOCS3 (Fig. 4A) and was not modified by the addition of anti-IFN-γ mAb (data not shown). In contrast, the amount of STAT3 phosphorylation was considerably higher in response to IL-6 in cells lacking SOCS3 (Fig. 4A). On analyzing IDO expression by Western blot using rabbit polyclonal IDO-specific Ab, we found that considerable expression of the enzyme protein was induced by IL-6 in cells lacking SOCS3 (Fig. 4B).
This was reflected by enhanced conversion of tryptophan to kynurenine, an occurrence unaffected by the presence of IFN-γ-neutralizing mAb (Fig. 4C). Therefore, IL-6 strongly activates STAT3 and tryptophan catabolism in DCs with silenced expression of SOCS3. IDO induction in DCs by IL-10 has been described previously to concur with enhanced STAT3 phosphorylation (24).

In additional experiments, we found that CD28-Ig, similar to IL-6, induced high kynurenine production in siRNA-SOCS3-treated DCs and that SOCS3 gene silencing did not enhance IFN-γ release in response to CD28-Ig (data not shown). It is also of interest that our previous studies have shown that CD28-Ig will increase SOCS3 transcriptional expression (11). Besides confirming this previous finding, in the present study, we found that CTLA-4-Ig greatly diminished this expression at 2 h (Fig. 4D). Thus, early modulation of SOCS3 expression by CD28-Ig and CTLA-4-Ig might be a working factor in the differential effects of the two fusion proteins.

In DCs lacking SOCS3, the IFN-like actions of IL-6 are an important mechanism of CD28-Ig activity

The data reported so far suggest that in DCs treated with CD28-Ig in the absence of SOCS3, IFN-γ signaling may be released from a critical inhibitory control and that IL-6 might contribute to IDO activation through the induction of IFN-γ-like responses. To ascertain the relative contributions of IFN-γ and IL-6 to the effects of CD28-Ig on CD8+ DCs lacking SOCS3, we adopted an experimental design similar to that of Fig. 3 and exposed siRNA-SOCS3-treated CD8+ DCs to CD28-Ig in the presence of Abs to IFN-γ or IL-6/IL-6R. The cells were then assayed for ability to block host priming to P815Ab by IL-6-conditioned CD8+ DCs (Fig. 5). The results showed that neutralization of IFN-γ and blockade of IL-6 activity were each capable of negating the induction of a tolerogenic phenotype by CD28-Ig in DCs lacking SOCS3. Therefore, the IDO-activating, IFN-γ-like responses induced by IL-6 in vitro in SOCS3-deficient DCs are reflected by effects in vivo that contribute critically to the tolerogenic properties of CD28-Ig in this specific setting.

In contrast to the effects of CTLA-4-Ig treatment, DCs treated with CD28-Ig secrete IL-6 in addition to IFN-γ and do not undergo up-regulation of IDO expression. Blockade of IL-6 restores IDO function in CD28-Ig-treated DCs, indicating that IL-6 effects are dominant over those of IFN-γ (11, 12). The basis for the predominant induction of IL-6 in DCs treated with CD28-Ig remains unclear, yet our current data indicate that SOCS3 is a major discriminator of function in DCs exposed to IFN-γ or IFN-γ-inducing agents in the presence of IL-6. In addition, our data provide the first experimental evidence that IL-6 can express immunosuppressive or IFN-γ-like actions in DCs lacking SOCS3. A major implication of this finding is that, in the absence of SOCS3, the functional consequence of prolonged IL-6 signaling is not simply unrestrained induction of IL-6-responsive genes but the activation of a different set of genes. Because DCs are chief regulators of the balance between tolerance and immunity, the finding that SOCS3 influences IL-6 transcriptional program in those cells may be relevant to the recognition of physiopathologic conditions in which SOCS3 could be poorly expressed (7) as well as to the implementation of novel immunotherapy protocols targeting the CD28/B7 costimulatory axis.

Disclosures
The authors have no financial conflict of interest.

References


