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SOCS3 Deletion in B Cells Alters Cytokine Responses and Germinal Center Output

Sarah A. Jones,*†,1 Christine A. White,*†,1 Lorraine Robb,*† Warren S. Alexander,*† and David M. Tarlinton*†

B cell behavior is fine-tuned by internal regulatory mechanisms and external cues such as cytokines and chemokines. Suppressor of cytokine signaling 3 (SOCS3) is a key regulator of STAT3-dependent cytokine responses in many cell types and has been reported to inhibit CXCL12-induced retention of immature B cells in the bone marrow. Using mice with SOCS3 exclusively deleted in the B cell lineage (Socs3<sup>Δ/Δ</sup>mb1cre<sup>+</sup>), we analyzed the role of SOCS3 in the response of these cells to CXCL12 and the STAT3-inducing cytokines IL-6 and IL-21. Our findings refute a B cell-intrinsic role for SOCS3 in B cell development, because SOCS3 deletion in the B lineage did not affect B cell populations in naive mice. SOCS3 was strongly induced in B cells stimulated with IL-21 and in plasma cells exposed to IL-6. Its deletion permitted excessive and prolonged STAT3 signaling following IL-6 stimulation of plasma cells and, in a T cell-dependent immunization model, reduced the number of germinal center B cells formed and altered the plasma cells exposed to IL-6. These data demonstrate a novel regulatory cytokine signaling circuit in plasma cells, providing, to our knowledge, the first evidence of how these long-lived, sessile cells respond to the external signals that mediate their longevity. The Journal of Immunology, 2011, 187: 000–000.

S uppressor of cytokine signaling 3 (SOCS3) is a negative feedback inhibitor of cytokine signaling through the JAK–STAT pathway. Signal attenuation is achieved via inhibition of signaling intermediates, either by direct or competitive binding or by engaging ubiquitination machinery and targeting activated JAKs, STATs, or cytokine receptors for proteasomal degradation (1). SOCS3 is a transcriptional target of STAT3 and, in turn, promotes the proteasomal degradation of phosphorylated STAT3 in a negative feedback loop (2–5). As IL-6 and IL-21 signal via STAT3 and have been described as important determinants of B cell and plasma cell survival and proliferation (6–8), we sought to determine the consequences of SOCS3 deletion in the B cell lineage, both on the development of B cells and plasma cells in the steady state and in response to challenge with Ag.

SOCS3 has been ascribed a role in early B cell development, specifically in reducing CXCL12-induced FAK phosphorylation and adhesion to VCAM-1 in the bone marrow (BM) (9). Using mouse mammary tumor virus (MMTV)-cre-mediated SOCS3 deletion, these authors showed a 2-fold increase in the number of immature B cells in the BM in the absence of SOCS3. The expression of cre recombinase under control of the MMTV promoter, however, causes deletion of floxed genes not only in B and T cells but also in some epithelial and secretory cell types as well as megakaryocytes and erythroid cells (10). B cell-related effects observed in this study may therefore be secondary consequences of deletion of SOCS3 in cells other than B cells. An unequivocal B cell-intrinsic role for SOCS3 in B cell development has not yet been determined.

SOCS3 may also influence B cell activation and differentiation in response to antigenic stimulation. Typical B cell responses to T cell-dependent Ags usually occur within the germinal center (GC) (11). The GC reaction is a coordinated process in which activated B cells migrate between the dark zone and the light zone. The dark zone is a site of intense B cell proliferation where the predominant chemokine is CXCL12 (12). Following bursts of proliferation and somatic hypermutation (SHM), B cells become responsive to CXCL13, which directs them out of the dark zone and toward the light zone (12, 13). In this study, follicular dendritic cells and T follicular helper (T<sub>fh</sub>) cells promote the selection and survival of B cell clones ( termed centrocytes) with high affinity for Ag (13). T<sub>fh</sub> cells secrete IL-21 (14, 15), which has direct effects on B cells (16, 17). IL-21 activates STAT3 in B cells to drive their expression of Bcl-6 (18), which occurs also in GC B cells, halting GC dissolution while B cells in the GC undergo affinity maturation (16, 17). Loss of IL-21, Bcl-6, or STAT3 impairs B cell responses to T cell-dependent Ags (7, 16–19). Thus, the responsiveness of GC B cells to chemokines and cytokines is essential for balancing proliferation and differentiation, as it is for B cell preservation.

To examine the cell-intrinsic roles of SOCS3 in regulating B cell and plasma cell development and behavior, we generated mice with SOCS3 deleted exclusively in the B cell lineage by crossing mice with floxed alleles of SOCS3 (20) with mice expressing cre recombi-
binase from the mb1 locus (21). The mb1 gene encodes CD79α, an intracellular component of the BCR, which is expressed exclusively in B cells beginning at the pro-B cell stage (21). Our data refute the finding that SOCS3 expression in immature B cells is a prerequisite for their timely exit from the BM (9) and shows that SOCS3 regulates both the maintenance of the GC and IgE affinity maturation during a T cell-dependent immune response.

Materials and Methods

**Mice**

Mice with floxed (20) or null (22) alleles of Socs3 were crossed with mice expressing cre recombinase under the mb1 promoter (21) or the MMTV promoter (10). All mice were on a C57BL/6 background and were maintained in a conventional animal facility. All procedures were performed in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Melbourne Health Animal Ethics Committee.

**Abs and flow cytometry for identification of B cell subsets**

Single-cell suspensions from BM or spleen were treated to lyse RBCs and stained with Ab conjugates for flow cytometry, including anti-CD19 (clone ID3), anti-CD21 (clone 7G6), anti-CD23 (clone B3B4), anti-B220 (clone RA3-6B2), anti-CD5 (clone 53-7.3), and anti-Synd-1 (clone 281; CD138), all purified and conjugated in our laboratory or purchased from BD Pharmingen. Avidin-Cy5 was obtained from Southern Biotechnology Associates. B cell subsets were identified based on surface marker expression. B cells as B220+IgM+IgD- (a population that may also contain some macrophage precursors, dendritic cells, and NK cells), immature B cells as B220-IgM-IgD-, and recirculating B cells as B220+IgM-IgD-. In spleen, transitional 1 (T1) B cells were identified as B220+IgM+IgD-CD21 CD23-, transitional 2 (T2) B cells as B220+IgM+IgD+CD21 CD23+, marginal zone B cells as B220+IgM+IgD+CD21+CD23- marginal zone B cells as B220+IgM+IgD+CD21+CD23+. Plasma cells were identified as B220lowIgD+.

Flow cytometry was performed on an LSR II or FACSCalibur (BD Biosciences) cytometer, and data on at least 10^4 viable cells, determined by propidium iodide exclusion, were collected. Live cells were sorted on the basis of propidium iodide exclusion on a FACS Diva (BD Instruments) or MoFlo (DakoCytomation) cytometer.

**Quantification of gene expression**

To measure cytokine induction of Socs3, IL-21R, IL-6Rα, or Bcl-6 mRNA expression, cells were FACS sorted as above and then stimulated with either 10 ng/ml recombinant mouse IL-21 (a gift from Zymogenetics) or 10 ng/ml IL-6 [supernatant from transfected hybridoma cell line, optimal concentration determined by cell culture (23)]. Total RNA was isolated from sorted B cells or plasma cells using an RNeasy Mini Kit (Qiagen) and quantified by spectrophotometer at 260 nm absorbance. With 100-600 ng total RNA (with equal amounts for comparable samples and the calibrator sample), cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The calibrator sample for expression of Socs3 and IL-6Rα was total RNA isolated from murine ES cells stimulated in vitro with IL-2 with FITC for 30 min. The calibrator sample for expression of Bcl-6 total RNA was isolated from murine ES cells stimulated in vitro with IL-4 with propidium iodide for 30 min. The calibrator sample for expression of Bcl-6 was total RNA isolated from wild-type skeletal muscle. The primer set for expression of IL-21R was FACS-sorted wild-type unstimulated B cells (B220-Synd-1^−). The negative control was the calibrator sample reaction without reverse transcriptase.

Real-time quantitative PCR (QPCR) was performed in triplicate or quadruplicate using TaqMan Gene Expression Assays (Applied Biosystems) for Socs3 (Assay ID: Mm01249143_g1), IL-21R (Assay ID: Mm00060319_m1), IL-6Rα (Assay ID: Mm00439653_m1), Bcl-6 (Assay ID: Mm00477633_m1), or the endogenous control hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) (Assay ID: Mm01318743_m1), using an Applied Biosystems 7900HT sequence detection system. Data were analyzed using SDS 2.2 software (Applied Biosystems) and the Relative Quantification (ΔΔCT) method. Data are shown as mean relative expression of Socs3, normalized for HPRT1 expression, with comparison to the calibrator sample.

**Detection of intracellular phosphorylated STAT3**

Detection of phosphorylated STAT3 by flow cytometry was achieved using the STAT3 (pY705)-Alexa Fluor 647 Ab and Phosflow reagents (BD Pharmingen) according to the manufacturer’s instructions. Flow cytometry was performed on an LSR II or FACS Calibur (BD Biosciences) cytometer, and data were collected on at least 10^4 viable cells, determined by propidium iodide exclusion.

**Transwell migration assays**

Sorted cells were resuspended in RPMI 1640 medium plus 5% FCS at a known cell concentration, which varied between cell populations depending on sort efficiency. This cell suspension (600 μl) was pipetted into the top wells of a 4-μm-pore Transwell plate (Corning Life Sciences) that were then placed into the lower wells containing 100 μl of either medium alone or 0.4 μg/ml CXCL12 (PeproTech). In some cases, cells were concurrently stimulated with 10 μg/ml F(ab’)_2 goat anti-mouse IgM (Jackson ImmunoResearch Laboratories). The plates were incubated at 37°C with 10% CO₂ for 4 h. The top wells were then removed and a known number of BD Calibrite beads (BD Biosciences) added to each lower well. This allowed the number of cells that had migrated through the pores to the lower wells to be accurately measured by flow cytometry.

**Immunization**

Mice were challenged with the model T cell-dependent Ag (4-hydroxy-3-nitrophenyl)acetyl-κ-keyhole limpet hemocyanin (NP-KLH) (Cambridge Research Biochemicals, Cleveland, U.K.), precipitated in alum. The Ag was injected (100 μl containing 100 μg Ag) i.p., and mice were kept on a heat pad and monitored for 24 h (24). Immunized mice were then bled retro-orbitally or mandibularly each week, and immune serum was isolated and tested for the presence and affinity of NP-specific Ab by ELISA as described below.

**Detection of NP-specific GC and memory B cells in the spleen**

For ELISA, serum was isolated from non–heparin-treated blood by allowing blood to clot for 4 h at 4°C, then centrifuging at 13,500 rpm for 7 min. The supernatant serum was removed and stored at −20°C. Serum Ig concentration was measured using ELISA, and Ig-secretion cells were counted by ELISPOT as described previously (25).

For capture of Ag-specific IgG1, NP coupled to BSA at either a high (NP17-BSA) or low (NP-BSA) haptenation rate was used as a plate coat for ELISAs or ELISPOTs, as described previously (24). Detection of anti-NP Abs of different isotypes was achieved using isotype-specific secondary Abs (anti-IgM, anti-IgG1, or anti-IgE) directly coupled to HRP (Southern Biotechnology Associates).

**Cell culture**

Sorted spleen B cells (CD19^+>) were cultured in triplicate at 5 × 10^6 or 3 × 10^6 cells in 200 μl RPMI 1640 medium plus 5% FCS plus 100 μM 2-ME with an optimal concentration of baculovirus-derived CD40L (determined by cell culture), IL-4 (100 U; PeproTech), and IL-21 (10 ng/ml) for 4 d at 37°C with 10% CO₂. Following this culture period, clones were vortexed twice for 10 s then incubated at room temperature for 20 min in the dark before analysis on a Canto II cytometer (BD Instruments). At least 10,000 events were recorded, and data were analyzed using FlowJo version 8.8.2 cell cycle analysis software (Tree Star).

**Statistical analyses**

Unpaired, two-tailed t tests were performed using Microsoft Excel software. Differences were deemed significant where p < 0.05.

**Results**

Socs3 expression is developmentally regulated in B cells and plasma cells and is further induced by IL-6 and IL-21.

Given that SOCS3 expression has been reported to increase as a B cell progresses through developmental stages in the BM (9), we
assessed the consequences of B cell-specific SOCS3 deletion on B cell development and differentiation. B cells of different subsets were sorted from BM and spleen, and SOCS3 mRNA expression was measured by QPCR (Fig. 1A). Pre-/pro-B cells and immature B cells in the BM contained a low level of SOCS3 mRNA, which was increased 3-fold (compared with pre-/pro-B cells) in recirculating mature B cells in the BM, 6-fold in follicular B cells and 4-fold in plasma cells in the spleen (Fig. 1A).

SOCS3 mRNA expression was dramatically induced by IL-21 in wild-type splenic B cells, increasing 25-fold after 30 min relative to B cells at rest (Fig. 1B). This effect was not observed in SOCS3Δ/Δ mb1cre splenic B cells, demonstrating efficient mb1-cre–mediated deletion of the loxP-flanked Socs3 locus in these cells (Fig. 1B). The lack of SOCS3 mRNA induction with IL-21 stimulation was not due to downregulation of IL-21R, because expression of IL-21R was not due to downregulation of IL-21R, because expression of IL-21R and IL-6Rα was both comparable in SOCS3+/+ and SOCS3Δ/Δ B cells, with or without IL-21 stimulation (Fig. 1C, 1D).

IL-21 was a much stronger inducer of SOCS3 mRNA expression than IL-6 in wildtype splenic B cells (Fig. 2A). Stimulation of plasma cells with IL-6 caused considerable induction of SOCS3 expression, the magnitude of which was greater than that of B cells stimulated with IL-6 (Fig. 2B).

**SOCS3 limits the intensity and duration of IL-6–mediated STAT3 signaling in plasma cells**

Despite the robust induction of SOCS3 mRNA expression upon stimulation with IL-21 (Figs. 1B, 2A), SOCS3 deficiency did not affect the magnitude or duration of STAT3 phosphorylation in B cells in response to IL-21 (Fig. 2C). Consistent with this finding, expression of the IL-21 target gene Bcl-6 was not affected by SOCS3 deletion (Fig. 2E). This indicates the involvement of alternative regulatory mechanisms in limiting IL-21–induced STAT3 activation and downstream target gene effects in B cells.

In plasma cells, interaction with BM stromal cells induces expression of IL-6, which may act to enhance plasma cell survival (26). As IL-6 signaling through STAT3 is regulated by SOCS3 in macrophages (2, 3, 5), we examined whether SOCS3 regulation of STAT3 phosphorylation occurred in plasma cells. SOCS3 deficiency in plasma cells resulted in elevated and sustained levels of phosphorylated STAT3 following their stimulation with IL-6 (Fig. 2D). To our knowledge, these data are the first to demonstrate that in plasma cells, as in macrophages, IL-6 induces phosphorylation of STAT3, causing expression of SOCS3, and that SOCS3 then attenuates signaling through this pathway by truncating STAT3 activity.

**Deletion of SOCS3 does not impair B cell development or response to CXCL12**

With MMTV-cre–mediated deletion, SOCS3 deficiency has been reported to cause a 2-fold accumulation of immature B cells in the BM (9), although this finding cannot be attributed to the loss of SOCS3 only in B cells. To isolate B cell-intrinsic effects of SOCS3 deficiency from those due to perturbations in the BM stromal environment, to which developing B cells are highly sensitive, B cell development was examined in Socs3Δ/+ mb1cre+ (SOCS3+/+) and Socs3Δ/+ mb1cre+ (SOCS3Δ−/−) mice. In these mice, no differences were observed compared with wild-type in the number or proportion of immature B cells or any other subset in the BM, and there were no differences detected in the sizes of splenic B cell populations (Fig. 3A). Moreover, in our hands, there were no changes detected in the number or proportion of immature B cells in the BM of Socs3Δ/+ MMTVcre+ (SOCS3Δ−/−) mice (Fig. 3B), in contrast to what has been reported previously (9).

**FIGURE 1.** SOCS3 is expressed in B cells and plasma cells and is further induced by cytokines. Data in each panel are expressed as mean relative expression of SOCS3, IL-21R, or IL-6Rα, normalized for HPRT1 expression, compared with the calibrator sample, as described in Materials and Methods. A and B, Bars represent mean ± SEM for cells from three to five mice. A, SOCS3 mRNA expression in FACS-sorted wild-type B cell populations: pre-/pro-B (BM, B220+IgM+ IgD−), immature (BM, B220+IgM+IgD−), recirculating (BM, B220+IgM+IgD−), follicular (spleen, B220+IgM+IgD−CD21+CD23−), and plasma cells (spleen, B220−IgM−IgD−Synd-1−). B, FACS-sorted naive B cells (B220+IgM−IgD−Synd-1−) from Socs3fl/fl mb1cre+ (SOCS3+/+) or Socs3fl/fl mb1cre+ (SOCS3Δ−/−) mice were stimulated with 10 ng/ml IL-21 for the indicated times in minutes, and SOCS3 mRNA expression was measured by QPCR (**p < 0.01 versus unstimulated SOCS3+/+). C, FACS-sorted naive B cells (B220−IgM−IgD−Synd-1−) from Socs3fl/fl mb1cre− (SOCS3+/+; n = 1) or Socs3fl/fl mb1cre− (SOCS3Δ−/−; n = 3) were stimulated with 10 ng/ml IL-21 for the indicated times in minutes, and IL-21R mRNA expression was measured by QPCR. D, FACS-sorted naive B cells (B220−IgM−IgD−Synd-1−) from Socs3fl/fl mb1cre− (SOCS3+/+; n = 1) or Socs3fl/fl mb1cre− (SOCS3Δ−/−; n = 3) were stimulated with 10 ng/ml IL-21 for the indicated times in minutes, and IL-6Rα mRNA expression was measured by QPCR.
Thus, our data do not support a role for B cell-expressed SOCS3 in regulating immature B cell development.

As well as its roles in polarizing the GC reaction and in directing plasma cells to the BM, the chemokine CXCL12 has been shown to retain developing B cells in the BM until they reach the immature stage, a process reported to be subject to SOCS3 regulation (9). To examine whether SOCS3 deletion only in B cells alters their responsiveness to CXCL12, BM B cell populations were sorted from $Socs3^{fl/fl}$mb1creΔ/Δ mice and $Socs3^{fl/fl}$mb1creΔ/+ mice as well as their isotype profile and Ig secretion. Despite our finding that SOCS3 regulates IL-6–induced STAT3 activation (Fig. 2D), deletion of SOCS3 did not alter the number or proportion of plasma cells in the BM and spleen of naive animals (Fig. 3A). The absence of SOCS3 and resultant increase in STAT3 signaling did not change the amount of IgG in the serum (Fig. 4).

Humans with STAT3 deficiency develop hyper-IgE syndrome (27) and deficiency of IL-21, which signals through STAT3, is also associated with hypersecretion of IgE (16, 28, 29). These observations suggest that alterations to STAT3 signaling in the absence of SOCS3 might perturb serum IgE levels. However, no differences were detected in IgE serum levels when SOCS3 was deleted

Thus, our data do not support a role for B cell-expressed SOCS3 in regulating immature B cell development.

FIGURE 2. SOCS3 deficiency alters the STAT3 response of plasma cells to IL-6 (A) FACS-sorted naïve B cells (B220$^+$Synd-1$^-$) from wild-type mice were stimulated with IL-21 or IL-6 for the indicated times in minutes, and SOCS3 mRNA expression was measured by QPCR ($^{*}p < 0.01$ versus IL-6 stimulation). B, FACS-sorted naïve B cells (B220$^+$Synd-1$^-$) or plasma cells (B220$^+$mb1creΔ/Δ) from wild-type mice were stimulated with IL-6 for the indicated times in minutes, and SOCS3 mRNA expression was measured by QPCR ($^{*}p < 0.05$ versus 0 min). A and B, Bars represent mean ± SEM for cells from three to five mice. C, Whole spleen cell suspensions from wild-type mice (Socs3$^{+/-}$mb1cre$^+$; SOCS3Δ/Δ) or mice heterozygous for Socs3 in all other cell types but lacking both alleles of Socs3 in B cells (Socs3$^{+/-}$mb1cre$^-$; SOCS3Δ/Δ) were stimulated with IL-21 for the indicated times in minutes and then fixed, permeabilized, and stained for intracellular phosphorylated (p)STAT3 levels and surface markers. The pSTAT3 response of B cells (B220$^+$Synd-1$^-$) is shown. D, Whole spleen cell suspensions from Socs3$^{+/-}$mb1cre$^+$ (SOCS3Δ/Δ) or Socs3$^{+/-}$mb1cre$^-$ (SOCS3Δ/Δ) mice were stimulated with IL-6 for the indicated times in minutes and then fixed, permeabilized, and stained for intracellular pSTAT3 levels and surface markers. The pSTAT3 response of plasma cells (B220$^+$Synd-1$^-$) is shown. C and D, The responses of cells from one to three mice are shown, and the data are representative of at least three independent experiments. E, FACS-sorted naïve B cells (B220$^+$Synd-1$^-$) from Socs3$^{+/-}$mb1cre$^+$ (SOCS3Δ/Δ; n = 1) or Socs3$^{+/-}$mb1cre$^-$ (SOCS3Δ/Δ; n = 3) mice were stimulated with 10 ng/ml IL-21 for the indicated times in minutes, and Bcl-6 mRNA expression was measured by QPCR.
in the B lineage, and there were no other significant differences in the serum levels of other Ab isotypes (Fig. 4).

**FIGURE 3.** SOCS3 deficiency does not alter B cell development or migration. B cells in the BM were identified as pre-pro-B (B220*IgM-IgD-), immature (B220*IgM*IgD-), recirculating (B220*IgM*IgD+), or plasma cells (B220-2*Synd-1*), and B cells in the spleen were identified as T1 (B220*IgM*IgD-CD21-CD23-), T2 (B220*IgM*IgD+CD21hi CD23hi), marginal zone (B220*IgM*IgD-CD21hi CD23hi), follicular (B220*IgM*IgD-CD21hi CD23hi), or plasma cells (B220-2*Synd-1*). A, Frequencies of B cell populations in the BM and spleen were determined by FACS in wild-type mice (Socs3+/+; SOCS3+/+), mice lacking one allele of Socs3 in B cells (Socs3fl/fl; Socs3+/+; SOCS3+/+), or mice heterozygous for Socs3 in all other cell types but lacking both alleles of Socs3 in B cells (Socs3fl/fl; Socs3fl/fl; SOCS3+/+). B, BM B cell populations were determined by FACS in wild-type mice (Socs3+/+; MMTVcre; SOCS3+/+), mice in which Socs3 was deleted using MMTV-cre (Socs3fl/fl; MMTVcre; SOCS3+/+), or mice in which Socs3 was deleted using MMTV-cre (Socs3fl/fl; MMTVcre; SOCS3+/+). C, B cell populations were sorted by FACS from the BM of Socs3+/+; MMTVcre (SOCS3+/+) or Socs3fl/fl; MMTVcre (SOCS3fl/fl) mice and subjected to Transwell migration assay where specific migration toward CXCL12 was determined over a 4-h period. There were no statistically significant differences in migration toward CXCL12 in SOCS3fl/fl compared with SOCS3+/+ for any of the B cell subsets tested. In each panel, bars represent mean ± SEM for cells from three to five mice.

**SOCS3 in B cells sustains the GC reaction in response to T cell-dependent immunization**

There is significant interest in the roles of IL-21 and STAT3 signaling in maintaining the GC reaction and promoting appropriately timed plasma cell differentiation (7, 16, 17). As our data showed that SOCS3 is induced upon IL-21 stimulation of B cells, we examined the effect of SOCS3 deficiency on the humoral response to immunization with the model GC-inducing Ag NP-KLH.

In a GC, T cells provide costimulatory factors including CD40L, IL-4, and IL-21 to B cells, and these factors sustain GC B cell survival and expansion (11, 16, 17). Stimulation of splenic B cells with the T cell costimulatory factors CD40L plus IL-4 and IL-21 in vitro resulted in fewer B cells after 4 d when SOCS3 was absent (Fig. 5A). To determine whether this translated to an in vivo deficiency in GC B cell numbers, we examined the effect of SOCS3 deletion on the number of GC B cells produced after immunization with NP-KLH. GC B cells were enumerated by flow cytometry by identifying (IgM, IgD, Synd-1, and Gr-1) negative, B220+ B cells that had switched to IgG1, possessed the ability to bind NP, and had not yet restored expression of CD38 (Fig. 5B).

The proportions of GC B cells in the G2 and S phases of the cell cycle at day 8 of the response were not altered by SOCS3 deficiency (Fig. 5C), showing that SOCS3 does not affect early proliferation of GC B cells. Supporting this, the number of SOCS3-deficient GC B cells was equal to wild-type on day 10 after immunization (Fig. 5D). Later in the response, however, GC B cells lacking SOCS3 were not sustained, and their numbers had diminished in comparison with wild-type by day 28 (Fig. 5D).

GC output includes both memory B cells and plasma cells that secrete isotype-switched, high-affinity Ab against the challenging Ag. No role was found for B cell-expressed SOCS3 in the production of IgG1 memory B cells during the early or late phases of the response (Fig. 6A), and the numbers of IgG1 plasma cells in BM and spleen were not significantly altered by SOCS3 deletion (Fig. 6B). In the BM and spleen, SOCS3 deletion had no significant effect on the numbers of anti-NP IgG1 plasma cells at day 28 (Fig. 6B), and this was reflected in the serum levels of anti-NP IgG1 being equivalent to wild-type at the same time point (Fig. 7B). Affinity maturation of NP-specific IgG1-secreting plasma cells in BM and spleen was not affected by SOCS3 deficiency, as shown by the normal affinity maturation of anti-NP IgG1 plasma cells that developed in the absence of SOCS3 (Fig. 6C). Taken together, these data show that the defect in SOCS3-deficient GC B cell maintenance does not translate into a defective IgG1 response.

In parallel with GC formation, NP-KLH normally induces extrafollicular proliferation of immature plasma cells that produce a peak of NP-specific IgM around 10 d after immunization (30, 31). This early Ag-specific IgM production was significantly diminished by SOCS3 deletion (Fig. 7A), indicating that prolonged STAT3 phosphorylation may skew the response away from the
extrafollicular IgM course. No bias was detected, however, toward Ag-specific IgG1 production by SOCS3-deficient plasma cells during the early phase of the response (Fig. 7B).

An additional consequence of immunization with NP-KLH in alum is the further switching of a small number of Ag-specific B cells to IgE, a process that appears to be regulated by IL-21 signaling in mice (28). The appearance of anti-NP IgE in serum of mice lacking SOCS3 in the B cell lineage initially occurred to the same extent as wild-type but surpassed the wild-type level as the response progressed (Fig. 7C). This was particularly apparent when the analysis was restricted to anti-NP IgE of high affinity, which continued to increase in the SOCS3-deleted animals after reaching a plateau in wild-type mice (Fig. 7C).

Taken together, these data reveal a B cell-intrinsic role for SOCS3 in maintaining GC B cell numbers during the late phase of a T cell-dependent Ab response. We have also shown that SOCS3 modulates the production of Ag-specific IgM and IgE in response to immunization with a T cell-dependent Ag, but its deletion has no significant effects on the IgG1 response.

**Discussion**

Fine regulation of the formation and behavior of B cells and their responsiveness to various immune environments are achieved through the activity of external factors including cytokines and chemokines. Several of these factors act via a STAT3-dependent signal transduction pathway. Because SOCS3 is a key feedback inhibitor of STAT3 signaling, particularly in response to IL-6 stimulation of macrophages (2, 3, 5), we sought to determine whether this inhibitory system extended to the B cell lineage and whether deletion of SOCS3 would impact facets of B cell behavior that are influenced by STAT3-inducing stimuli.

SOCS3 expression was low in pre-/pro-B cells and immature B cells and increased as the cells matured, supporting previously published data (9). Using MMTV-cre–mediated SOCS3 deletion, Le et al. (9) found that SOCS3 inhibited adhesion responses of developing B cells in the BM and allowed their timely egress to the periphery. SOCS3 deletion was reported to cause a 2-fold increase in the number of immature B cells retained in the BM (9). The use of MMTV-driven cre recombinase expression, however,
will result in SOCS3 deletion in a number of hemopoietic cell types, including B and T cells, megakaryocytes, and erythroid cells (10). Although this level of deletion avoids the embryonic lethality that occurs in mice lacking SOCS3 in all tissues (22, 32), $Socs3^{fl/fl} MMTVcre^{+}$ mice show evidence of disrupted immune function as a result of altered chemokine secretion and neutrophil responses (33, 34). We have shown that when SOCS3 is deleted exclusively in B cells using $mb1$-cre, there is no accumulation of immature B cells in the BM. In addition, in our colonies of $Socs3^{fl/fl} MMTVcre^{+}$ mice, numbers of immature B cell populations were not different from wild-type (Fig. 3B). Therefore, evidence presented in this article is not consistent with the finding that SOCS3 deficiency in immature B cells prevents them from leaving the BM (9) and points to a role for SOCS3 in other cell types as the explanation for this phenomenon.

In mature B cells, the level of SOCS3 mRNA expression was around half that produced by ES cells stimulated with LIF. Upon exposure to IL-6, SOCS3 expression by B cells increased 5-fold over 60 min, whereas IL-21 raised SOCS3 transcript levels 25-fold within 30 min. A role for IL-21 has recently been described in maintaining B cells as they progress through the GC reaction (16, 17). GC B cells are subject to major assault on the integrity of genes at the Ig loci during the process of SHM and class-switch recombination. While this process occurs and the cell cycle is halted, IL-21 signaling prevents apoptosis of GC B cells (16, 17). This is thought to occur mainly via the induction of Bcl-6 by IL-21–stimulated STAT3 activation (18, 35, 36), although STAT3 deficiency in B cells does not fully recapitulate the phenotype of mice with impaired IL-21 signaling (7, 16, 17).

Our data show that SOCS3 deficiency does not alter STAT3 phosphorylation or Bcl-6 expression following IL-21 stimulation of mature B cells, indicating that alternative mechanisms can modulate signaling in this pathway when SOCS3 is absent. This is not the case for the IL-6 pathway in plasma cells, where we found that SOCS3 was required for the regulation of STAT3 phosphorylation—a phenomenon that is also observed in macrophages (2, 3, 5).

Our data reveal previously unknown roles for IL-21–induced SOCS3 in promoting B cell proliferation and negatively regulating IgE switching. In vitro stimulation of B cells with the T cell costimulatory factors CD40L, IL-4, and IL-21 produced fewer B cells after 4 d when SOCS3 was absent. Although this indicated that early GC B cell proliferation might be impaired in the absence of SOCS3, there was no effect of SOCS3 deletion on either the cell cycle status of ex vivo GC B cells on day 8 of the response or on in vivo GC B cell numbers on day 10.

Complete deletion of IL-21 in mice increases the levels of both total and Ag-specific IgE in serum (16, 28, 37), and STAT3 deficiency in humans causes hyper-IgE syndrome (27). As deletion of SOCS3 in B cells also increases the production of Ag-specific IgE, all three molecules appear to be negative regulators of IgE switching. Class-switch recombination to Ig$\varepsilon$ is division linked (38); therefore, the effects of IL-21, STAT3, and SOCS3 on IgE production may be via limiting the small fraction of B cells that continue to proliferate and switch to IgE.

IL-21 reduces B cell proliferation and blocks excess IgE production in response to a T cell-dependent Ag (16, 28, 37). In the absence of SOCS3, GC B cells may be more sensitive to IL-21 signals, causing an increase in IgE plasma cell production and
a reduction in cell proliferation at the later stages of the GC reaction. Enhanced STAT3 signaling, which is associated with IgG1 switching (7), might cause a concomitant increase in the proportion of GC B cells switching to IgG1. On balance, these two factors could result in an apparently normal number of IgG1–memory B cells and plasma cells exiting the GC in the absence of SOCS3.

Post-GC plasma cell formation requires STAT3 (7), which we have shown to be activated when plasma cells are exposed to IL-6. SOCS3 has a nonredundant role in regulating this pathway as its loss increases the duration of STAT3 phosphorylation. In addition, it has been shown that excess IL-6 signaling promotes a surplus of plasma cells (39–41). Alterations at the biochemical level, however, do not translate to cellular changes in SOCS3-deficient plasma cells in the steady state. The number of plasma cells in the spleen and BM was not affected by SOCS3 deficiency, and the general IgG1 plasma cell response to NP-KLH immunization was not substantially altered by the loss of SOCS3. The production of Ag-specific IgM, however, was significantly lower when SOCS3 was absent. Whether this can be attributed to a role for SOCS3 in sustaining the proliferation of B cells destined for extrafollicular plasmablast responses or in inhibiting switching away from IgM is unclear.

By deleting SOCS3 exclusively in B cells, we have demonstrated the cell-intrinsic roles of SOCS3 in B cell and plasma cell development and behavior. Although SOCS3 was induced in B cells and plasma cells with IL-21 and IL-6 stimulation, respectively, its deletion did not have major cellular consequences in the steady state. Upon immunization with a T cell-dependent Ag, however, the maintenance of SOCS3-deficient GC B cells was impaired. A novel role for SOCS3 was found in the formation of Ag-specific IgM early in the GC reaction and in inhibiting the production of IgE in the later stages of the response. These findings may have implications for the treatment of allergies in which IgE production is thought to be pathophysiologically significant.

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Disclosures

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