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A Physiological Function of Inflammation-Associated SerpinB2 Is Regulation of Adaptive Immunity

Wayne A. Schroder,* Thuy T. T. Le,* Lee Major,* Shayna Street,[†] Joy Gardner,* Eleanore Lambley,* Kate Markey,* Kelli P. MacDonald,* Richard J. Fish,[‡] Ranjeny Thomas,[†] and Andreas Suhrbier*

SerpinB2 (plasminogen activator inhibitor-2) is widely described as an inhibitor of urokinase plasminogen activator; however, SerpinB2$^{-/-}$ mice show no detectable increase in urokinase plasminogen activator activity. In this study, we describe an unexpected immune phenotype in SerpinB2$^{-/-}$ mice. After immunization with OVA in CFA, SerpinB2$^{-/-}$ mice made ≈6-fold more IgG2c and generated ≈2.5-fold more OVA-specific IFN-γ–secreting T cells than SerpinB2$^{+/+}$ littermate controls. In SerpinB2$^{+/+}$ mice, high inducible SerpinB2 expression was seen at the injection site and in macrophages low levels in draining lymph nodes and conventional dendritic cells, and no expression was seen in plasmacytoid dendritic, B, T, or NK cells. SerpinB2$^{-/-}$ macrophages promoted greater IFN-γ secretion from wild-type T cells in vivo and in vitro and, when stimulated with anti-CD40/IFN-γ or cultured with wild-type T cells in vitro, secreted more Th1-promoting cytokines than macrophages from littermate controls. Draining lymph node SerpinB2$^{-/-}$ myeloid APCs similarly secreted more Th1-promoting cytokines when cocultured with wild-type T cells. Regulation of Th1 responses thus appears to be a physiological function of inflammation-associated SerpinB2; an observation that may shed light on human inflammatory diseases like pre-eclampsia, lupus, asthma, scleroderma, and periodontitis, which are associated with SerpinB2 polymorphisms or dysregulated SerpinB2 expression. *The Journal of Immunology*, 2010, 184: 2663–2670.

S erpinB2 is a member of the Clade B or OVA-like serine protease inhibitor (ov-serpin) subgroup of the serpin superfamily, which includes proteinase inhibitors 6, 8, and 9, MENT, Bomapin, and maspin. Ov-serpins lack a classical secretory signal peptide and are frequently found to have cytoplasmic or nucleocytoplasmic distributions and functions (1, 2). SerpinB2 is induced during many inflammatory processes and infections (3–7) and is one of the most upregulated proteins of activated monocytes/macrophages representing up to 1% of total protein (7, 8). SerpinB2 can also be induced to a lesser extent in fibroblasts and endothelial cells and is constitutively expressed by differentiating keratinocytes, placental trophoblasts (3), adipocytes (9), and a range of tumors (4, 10).

SerpinB2 is also known as plasminogen activator inhibitor (PAI) type 2 and is widely described as an inhibitor of the extracellular urokinase plasminogen activator (uPA), because SerpinB2 can be shown to inhibit uPA in vitro (3, 4). The majority of the >860 publications listed in PubMed on SerpinB2/PAI-2 have thus assumed that the principle role of this serpin is inhibition of uPA, although a number of reports have indicated that certain activities associated with SerpinB2 expression appear unrelated to uPA inhibition (2, 6, 9, 11–14). Although SerpinB2 can inhibit uPA in vitro, the evidence that this represents a physiological function for SerpinB2 in vivo is not compelling. SerpinB2$^{-/-}$ mice show no apparent defects in uPA activity, and mice deficient in both SerpinB2 and PAI-1 show no additional defects in uPA activity over PAI-1$^{-/-}$ mice (15). Furthermore, covalent uPA-SerpinB2 complexes, which are easily generated in vitro, have not been unequivocally demonstrated in vivo, whereas uPA-PAI-1 complexes have been readily detected (16). The majority of SerpinB2 is also retained intracellularly, because it is inefficiently secreted (3). The physiological function of inflammation-associated SerpinB2 thus remains unclear (4).

Clinically, dysregulated SerpinB2 expression or SerpinB2 polymorphisms have been associated with a number of diseases involving inflammation. Decreased expression is associated with pre-eclampsia (17), whereas expression is upregulated in asthma (18), periodontal disease (19), and hyperkeratotic corn tissue (20). Increased SerpinB2 expression in certain cancers is also associated with improved prognosis (10). SerpinB2 polymorphisms have also been associated with antiphospholipid syndrome (21), lupus (22), and myocardial infarction in some (23) but not other studies (24). SerpinB2 has been reported to have a bewildering array of activities including regulation of monocyte and keratinocyte proliferation and differentiation (13, 25, 26), inhibition of apoptosis in some (5, 14, 27) but not other settings (28), inhibition of necrosis (29), inhibition of the IL-1β converting enzyme (30), inhibition of retinoblastoma protein degradation (31), and priming of IFN-α/β responses (6). SerpinB2 has been reported to bind annexins (32), the retinoblastoma proteins (2, 12), IFN response factor 3 (33), ZNF198/FGFR1 fusion kinase (34), proteasome subunit, β type 1 (35), and vitronectin (36). These numerous activities and the limited evidence supporting a role in uPA inhibition

leave no clear basis for understanding the clinical observations associated with SerpinB2.

Using SerpinB2$^{-/-}$ mice (15), we sought to gain insights into the physiological role of inflammation-associated SerpinB2. Only two phenotypes have so far been reported for SerpinB2$^{-/-}$ mice: increased susceptibility to multistage skin carcinogenesis (31) and increased adipocyte hypotrophy after a high-fat diet (9). Neither phenotype provides discernable insights into the clinical observations. In this study, using SerpinB2$^{-/-}$ mice that have been fully backcrossed onto a C57BL/6 background, we describe a hitherto unreported activity of SerpinB2. These mice produce more IgG2c and T cell IFN-γ (i.e., show an increased Th1 bias) after immunization with OVA formulated in CFA. The phenotype mapped to CD40 signaling in macrophages and draining lymph node myeloid APCs and suggests that a physiological role of SerpinB2 is suppression of Th1-promoting cytokine production by these cells. This observation is consistent with the clinical associations seen between SerpinB2 dysregulation and polymorphisms and a number of inflammatory diseases.

Materials and Methods
Mice

SerpinB2$^{-/-}$ mice backcrossed six times onto a C57BL/6 background were obtained from Dr. D. Ginsburg (University of Michigan Medical School, Ann Arbor, MI) (15) and were backcrossed onto the same background an additional six times. To genotype mice, DNA was extracted from tail tips and analyzed by multiplex PCR using Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO). The multiplex PCR used the primers: neo, 5′-CAGCCGAA-CTGTTCGCCAGG-3′; SerpinB2 (1), 5′-TGATAGGCGGGTTGTTTCTC-TGTGC-3′; and SerpinB2 (2), 5′-CCACTCCAGCAGAATGCCAGC-3′. The SerpinB2 (1) and SerpinB2 (2) primers amplify a 520-nt product from the wild-type allele and the neo and SerpinB2 (2) primers produce a 670-nt product from the targeted allele. After the final backcross, SerpinB2$^{+/-}$ animals were intercrossed, with the progeny used to establish SerpinB2$^{-/-}$ knockout and SerpinB2$^{+/+}$ littermate control colonies. C57BL/6 mice (Animal Resources Centre, Perth, Western Australia, Australia) were also used as control mice in some experiments.

Immunization and ELISA

Mice were immunized once s.c. with OVA (100 μg/mouse) dissolved in PBS formulated with CFA (Sigma-Aldrich) (1:1 v:v, final volume 50 μl), poly-inosinic:polycytidilic acid [poly(I:C)] (InvivoGen, San Diego, CA) (100 μg, final volume 50 μl), or 1826 CpG oligonucleotides (Sigma-Aldrich) (100 μg, final volume 50 μl). IgG2c (A) and IgG1 (B, C) titers were measured as in Fig. 1 (n = 5/group). After 3 wk, serum Ab titers were analyzed by ELISA using OVA-coated (10 μg/ml overnight) Maxisorb plates (Nalge Nunc International, Rochester, NY), biotin conjugated rat anti-mouse IgG2c (R19-15) and IgG1 (A85-1) (BD Pharmingen, Heidelburg, Germany), streptavidin-HRP (BioSource International, Camarillo, CA) and ABTS substrate (Sigma-Aldrich).

ELISPOT assays

Ex vivo ELISPOT assays were conducted using splenocytes as previously described (37) in the presence of 10 μg/ml OVA. Cultured ELISPOT assays were performed by culturing splenocytes in vitro for 6 d in 24-well plates (5 × 10^6 cells/well in 1 ml of medium) with 10 μg/ml OVA. On day 4, an additional 1 ml of medium was added to each well. On day 6, the cells were used in the ELISPOT assay. Spots were counted using the Bioreader 5000 (Bio-Sys, Karben, Germany). Where indicated, anti-CD3 (2C11) was used at 1 μg/ml instead of OVA. Background spots in the absence of anti-CD3 or OVA were subtracted. Resident peritoneal macrophages (RPMs) were generated by seeding lavaged cells onto plastic. Nonadherent cells were removed by washing after 3 and 24 h; adherent cells were trypsinized and seeded in duplicate into ELISPOT plates (5 × 10^4 cells/well). Splenic CD3$^+$,F4/80$^-$ T cells from C57BL/6 mice were isolated by FACS sorting (MoFlo; BD Biosciences, Franklin Lakes, NJ).

uPA assay and proliferation assays

Thioglycollate-elicited peritoneal macrophages (TEPMs) (obtained day 4 after i.p. injection of 2 ml of 3% thioglycollate) or RPMs were isolated by adherence, and 7 × 10^6 cells in 1 ml were cultured overnight with or without 100 ng/ml LPS (Escherichia coli O55:B5; Sigma-Aldrich). Supernatants were concentrated 10-fold using Microcon centrifugal filter unit with a 3000 m.w. cutoff (Millipore, North Ryde, New South Wales, Australia), and uPA activity was measured using the Mouse uPA activity assay kit (Innovative Research, Novi, MI). For proliferation assays, splenocytes from SerpinB2$^{-/-}$ and SerpinB2$^{+/+}$ mice were incubated in duplicate with 0.5 μg/ml anti-CD3 and/or 100 ng/ml LPS for 2 d, and proliferation was measured by MTS assay (Promega, Madison, WI).

FACS analysis

FACS analysis was undertaken using the FACSCalibur (BD Biosciences). Abs used where CD3/CD19 PE/FITC (Serotec, Kidlington, U.K.), F4/80 (FITC) (Serotec), CD11c (PE) (Serotec), MHC2 (PE) (BD Pharmingen), TCRα/β (FITC) (Caltag Laboratories, Burlingame, CA), CD4 (Tricolor) (Caltag Laboratories), and CD8β (PE). BD Fc Block (BD Biosciences) was used to block the FcR. Isotype controls were IgG2b (PE), IgG1 (FITC) (Biolegend, San Diego, CA), and IgG2b (FITC) (BD Pharmingen).

Real-time RT-PCR

The following were placed into RNA*later* Solution (Ambion, Austin, TX) for 24 h at 4°C: 1) excised injection site tissue (skin removed) and draining lymph nodes, 2) RPMs incubated with LPS (E. coli 055:B5 (Sigma-Aldrich) (100 ng/ml), phosphothiorate-modified CpG (2.5 μM, ODN 1826), or poly(I:C) (25 μg/ml) (InvivoGen) for the indicated time, or 3) splenic DCs purified by Optiprep density gradient (38) or the same DCs separated by FACS (MoFlo) into pDCs (CD11c^{mid}, 120G8/CD317$^+$) and cDC (CD11c^{hi}, 120G8/CD317$^-$) populations and treated as above. RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First strand total cDNA synthesis was performed in a 20-μl reaction containing 1 μg total RNA, 500 μM 2′-deoxynucleoside 5′-triphosphates, 200 ng random hexamer oligonucleotides, 1× Superscript First Strand buffer, 10 mM DTT, and 200 U Superscript III (Invitrogen). Real-time PCR analysis used the following nucleotide primers: for SerpinB2, 5′-CTGCTACCCGAAGGT-TCTG-3′ and 5′-GGAAGCAACAGGAGCATGC-3′, and for RPL13A (39), 5′-GAGGTCGGGTGGAAGTACCA-3′ and 5′-TGCATCTTGGCCTTTT-CCTT-3′. The amplification reaction mixture of 20 μl contained 0.1 μg randomly primed cDNA, 0.5 μM each primer pair, and 10 μl 2× Platinum SYBR Green Quantitative PCR Supermix-UDG (Invitrogen). Cycling conditions were as follows: one cycle of 50°C for 2 min and one cycle of 95°C for 2 min, followed by 45 cycles of 94°C for 5 s, 60°C for 10 s, and 72°C for 40 s. The real-time PCR was performed using a Rotor-Gene 3000 PCR machine (Corbett Research, Mortlake, New South Wales, Australia). The data were analyzed with Rotor-Gene Real Time Analysis software (Corbett Research). Each sample was analyzed in duplicate and normalized to RPL13A mRNA because mRNA expression for this housekeeping gene remains constant even under conditions of widespread gene induction (39).

Western blot analysis

Western blot analysis was undertaken as previously described (40) using anti-GAPDH (Chemicon International, Temecula, CA) and an anti-SerpinB2 Ab raised in rabbits against the CD loop region peptide, CEIGSYGITTR-NPEN, coupled to keyhole limpet hemocyanin and formulated in CFA/IFA.

DC analysis

Four color flow cytometry (41) was used to identify cDC (class II^{hi}/CD11c^{hi}) and pDC (PDCA1$^+$/CD11c^{int}) in spleens isolated from SerpinB2$^{-/-}$ and SerpinB2$^{+/+}$ littermate mice. cDC subset frequency was analyzed as described previously (38). Allogeneic T cell proliferation (41) was undertaken using purified cDCs (>98% pure) from SerpinB2$^{-/-}$ and SerpinB2$^{+/+}$ littermate mice and 10^5 wild-type allogeneic BALB/c T cells.

Adoptive transfer of macrophages

RPMs were obtained by peritoneal lavage (from 25 mice) and cultured for 3 h with nonadherent cells removed by washing. The cells were cultured for an additional 4 d with daily removal of nonadherent cells. On day 4, OVA (1 mg/ml) was added and the cells incubated overnight, and then LPS (1 μg/ml) was added for an additional 3 h. The cells were trypsinized and washed and injected i.v. into C57BL/6 mice (10^6 cells/mouse). After 10 d, splenocytes were isolated from injected mice and analyzed by ELISPOT assay.

Depletion of macrophages

Mice were injected on day −1 i.v. with 200 μl control liposomes or liposomes containing clodronate (42, 43). Spleen F4/80$^+$ macrophages were depleted by 86 ± 5.5% on day 0 (n = 4) (data not shown). OVA/CFA was injected day 0 in a parallel group of animals, and Ab and ex vivo ELISPOT assays were performed as described above.

Cytokine/chemokine analysis

Cytokines/chemokine levels were analyzed in supernatants using the BD Cytokine Bead Array Bioanalyzer System (BD Biosciences, North Ryde, New South Wales, Australia) as per the manufacturer's instructions. RPMs were isolated from SerpinB2$^{-/-}$ or SerpinB2$^{+/+}$ (littermate) mice ($n = 6$/group) by adherence and daily washing over 5 d. RPMs (5×10^4/96 well) from each mouse were treated with LPS (100 ng/ml), IFN-γ (20 IU/ml), and/or anti-CD40 (100 μg/ml) (FGK45; Alexis Biochemical, San Diego, CA) for 6 h, and supernatants were analyzed for cytokine/chemokine levels.

Cytokine/chemokine production by cells isolated from draining lymph nodes

Draining lymph nodes isolated day 4 from OVA/CFA-immunized SerpinB2$^{-/-}$ and SerpinB2$^{+/+}$ mice were collected and pooled for each group ($n = 16$/group). CD11c$^+$ DCs, CD11b$^+$ macrophages, and CD4$^+$ T cells were positively selected sequentially using the AutoMACS Pro (Miltenyi Biotec, North Ryde, New South Wales, Australia) and CD11c, CD11b, and CD4 microbeads as per manufacturer's protocols (Miltenyi Biotec). CD11c$^+$ and CD11b$^+$ cells at 2×10^4 cells/well were incubated with anti-CD3 (0.5 μg/ml) and 5×10^4 purified CD4$^+$ T cells for 72 h. Supernatants were collected and assayed for cytokine/chemokine levels as described above in triplicate.

Statistics

Statistical analysis was performed using SPSS for Windows (version 15.0, 2007; SPSS, Chicago, IL). For comparison of two samples, the t test was used when the difference in the variances was <4 and skewness was >-2 and kurtosis was <2; otherwise, the non parametric Mann-Whitney U test was used. For comparison of data involving multiple experiments, the nonparametric Kruskal-Wallis ANOVA was used.

Results

Th1 responses are increased after immunization of SerpinB2$^{-/-}$ mice with OVA/CFA

To determine the role of SerpinB2 in antigen-specific priming, SerpinB2$^{-/-}$ and control mice (SerpinB2$^{+/+}$ littermate or C57BL/6 mice) were immunized with OVA Ag formulated with CFA (OVA/CFA). In three separate experiments following immunization with OVA/CFA, SerpinB2$^{-/-}$ mice showed a mean 5.9-fold ($\pm$ SE 0.41) higher OVA-specific IgG2c titer than littermate controls. C57BL/6 mice gave essentially the same titers as the littermate controls (Fig. 1A). No significant differences were observed in IgG1 (Fig. 1B) or total IgG titers between SerpinB2$^{-/-}$ and control mouse groups (data not shown). The differences in IgG2c titres remained after OVA/CFA boosting, and similar differences were obtained when tetanus toxoid was substituted for OVA (data not shown).

OVA-specific T cell responses were analyzed after OVA/CFA immunization by ex vivo and cultured IFN-γ ELISPOT assays, which reflect the levels of effector memory and central memory cells, respectively, as discussed previously (37). SerpinB2$^{-/-}$ mice generated a $\approx$2.5-fold higher number of OVA-specific IFN-γ–secreting splenic T cells than control mice in both assays (Fig. 1C, 1D). No differences in cell numbers or IFN-γ mRNA levels was seen in FACS-sorted splenic NK cells (NK1.1$^+$, CD3$^-$, F4/80$^-$, and CD11c$^-$) from SerpinB2$^{-/-}$ and SerpinB2$^{+/+}$ mice 18 h after OVA/CFA vaccination (44) (data not shown), suggesting that SerpinB2 deficiency does not significantly affect NK cell activity after OVA/CFA vaccination.

The increased T cell IFN-γ responses in SerpinB2$^{-/-}$ mice are consistent with the increased IgG2c responses seen in these animals and indicate that SerpinB2 deficiency enhances Th1 responses after OVA/CFA immunization.

No changes in uPA activity, T cell proliferation, or draining lymph node cell composition in SerpinB2$^{-/-}$ mice

SerpinB2 is widely reported to inhibit uPA and macrophages are major producers of SerpinB2 (3). However, when TEPMs from SerpinB2$^{-/-}$ and SerpinB2$^{+/+}$ mice were analyzed for uPA activity, no significant differences in uPA activity were observed (Fig. 2A).
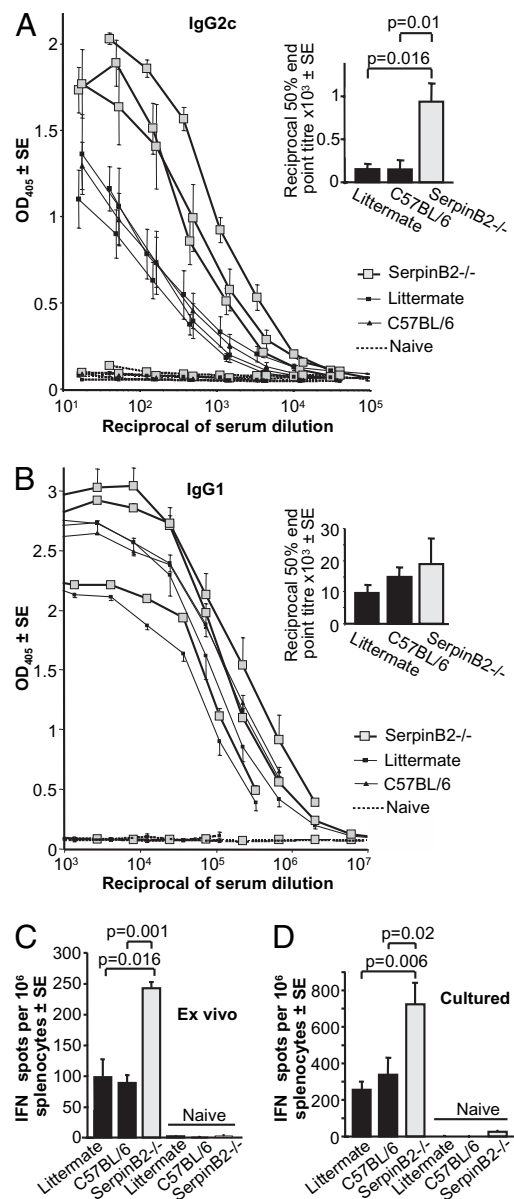
FIGURE 1. SerpinB2$^{-/-}$ mice produce more IgG2c and IFN-γ–secreting T cells after OVA/CFA immunization. A, SerpinB2$^{-/-}$ mice, SerpinB2$^{+/+}$ littermate controls and wild-type C57BL/6 mice were immunized with OVA/CFA. Serum IgG2c titers from three separate experiments are shown. Bar chart shows average 50% end point titers from all animals, and SerpinB2$^{-/-}$ mice generated an average 5.9-fold more IgG2c than littermate controls. Significance was determined using the Kruskal-Wallis ANOVA with a term for experiment. B, As for A, except that IgG1 titers are shown. C and D, Mice were immunized as for A, and after 3 wk, splenocytes were analyzed by ex vivo (C) and cultured (D) IFN-γ ELISPOT assays (SerpinB2$^{-/-}$, $n = 9$; littermate controls, $n = 5$; C57BL/6, $n = 10$). An average 2.6 (range, 2.1–2.8)-fold more IFN-γ–producing cells were generated in SerpinB2$^{-/-}$ mice compared with littermates and C57BL/6 mice. Significances were calculated using the Mann-Whitney U test.

uPA activity in the supernatants from cultures of RPMs was barely detectable and also showed no significant differences between SerpinB2$^{-/-}$ and SerpinB2$^{+/+}$ mice (data not shown).

uPA$^{-/-}$ mice have impaired Th1 and Th2 responses (45) potentially due to a general defect in T cell proliferation, illustrated by reduced responses to anti-CD3 (46). Any increase in uPA activity in SerpinB2$^{-/-}$ mice might thus be expected to promote T cell proliferation. However, no differences in T cell proliferation were
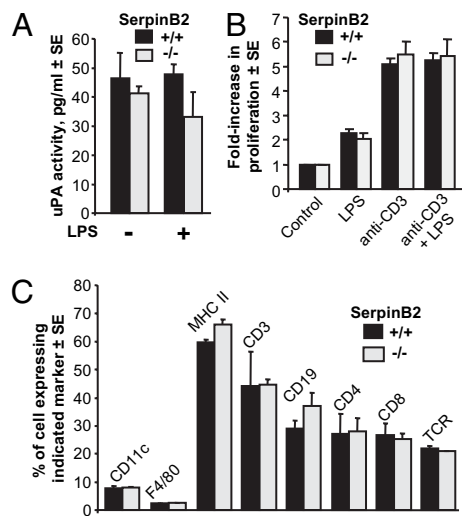
FIGURE 2. uPA activity, T cell proliferation, and lymph node composition. *A*, uPA activity in the supernatants of TEPMs after treatment with or without LPS (100 ng/ml) measured by capture ELISA (*n* = 4 mice/group). *B*, Proliferation of splenocytes from SerpinB2$^{-/-}$ and littermate controls after stimulation with LPS and/or anti-CD3 (average of three experiments ± SE). *C*, Percentage of cells expressing the indicated markers present in draining lymph nodes 2 d after immunization with OVA/CFA. The average of two experiments is shown, each using pooled draining lymph node cells from three mice.

observed between SerpinB2$^{-/-}$ and littermate control mice using anti-CD3 (Fig. 2*B*), even in the presence of LPS, which induces high SerpinB2 expression. Similar results were obtained when PHA was used (data not shown). SerpinB2$^{-/-}$ mice thus do not appear to have a general defect in T cell proliferation.

uPA is believed to be important for cell migration (3). However, cell composition in draining lymph nodes before (data not shown) and after vaccination (Fig. 2*C*), and the numbers of F4/80$^+$ macrophages at the injection site before and after CFA immunization were no different in SerpinB2$^{-/-}$ and SerpinB2$^{+/+}$ mice (data not shown).

Peripheral blood and spleen T cell, B cell, DC, platelet, and granulocyte cell numbers were also similar in naive SerpinB2$^{-/-}$ and SerpinB2$^{+/+}$ mice (data not shown). Taken together with previous studies using these animals (15), these data provide additional evidence for the apparent lack of differences in uPA activity in SerpinB2$^{-/-}$ mice.

SerpinB2 expression in vivo, and in macrophages, DCs, B cells, T cells, and NK cells

To gain a better insight into the mechanisms involved in the increased Th1 bias in SerpinB2$^{-/-}$ mice, we first characterized the dynamics of SerpinB2 expression in SerpinB2$^{+/+}$ mice after OVA/CFA immunization. Injection sites and draining lymph nodes were removed at different times and analyzed by real-time RT-PCR for SerpinB2 mRNA. One day after immunization, SerpinB2 mRNA levels were induced >150-fold at the injection site relative to unimmunized skin and declined over the subsequent days (Fig. 3*A*, injection site). SerpinB2 mRNA levels were also increased ≈2.5-fold in the draining lymph nodes, with levels declining over the subsequent days (Fig. 3*A*, lymph nodes). Thus, SerpinB2 is rapidly, but transiently, induced both at the injection site and the draining lymph nodes.

Although SerpinB2 is well-known to be expressed by activated macrophages (3), expression in DCs and B and T cells has not been explored extensively. SerpinB2 mRNA expression was thus analyzed using real-time RT-PCR in these cell populations. Of the different macrophage populations investigated, RPMs had the highest constitutive [consistent with previous observations (47)] and
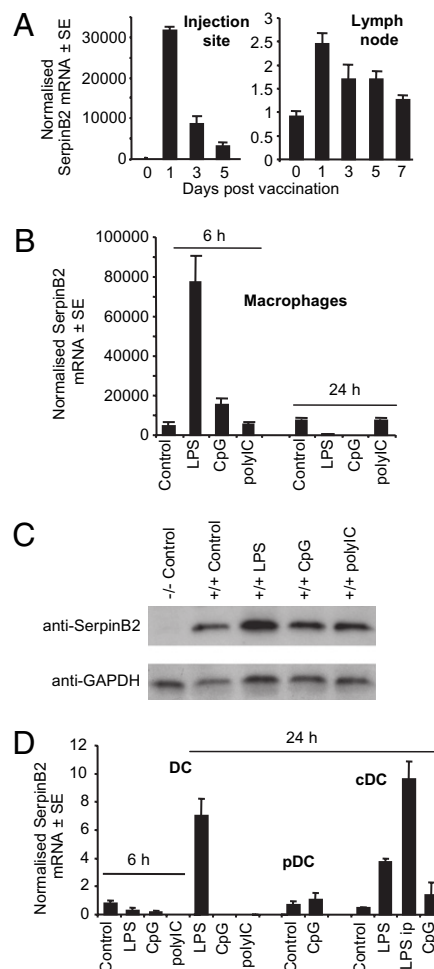
FIGURE 3. SerpinB2 expression. *A*, Real-time RT-PCR analysis of injection site and draining lymph node SerpinB2 mRNA levels (normalized to RPL13A) from wild-type mice at the indicated times after vaccination (*n* = 3/time point). Time zero represents unimmunized skin. *B*, Real-time RT-PCR analysis of SerpinB2 mRNA levels (normalized to RPL13A) in RPMs after treatment with medium (control), LPS (100 ng/ml), CpG (2.5 μM, ODN 1886), or poly(I:C) (25 μg/ml) for the indicated time. *C*, Western blot of the RPM populations described above using Abs specific for SerpinB2 and GAPDH. RPMs from SerpinB2$^{-/-}$ mice were included as a negative control (*first lane*). *D*, Real-time RT-PCR analysis of SerpinB2 mRNA levels (normalized to RPL13A) in splenic CD11c$^+$ DCs and pDCs and cDCs treated as in *B* for the indicated times. LPS (10 μg) was also injected i.v. and cDCs isolated after 24 h (LPS in vivo).

inducible SerpinB2 mRNA and protein expression levels. LPS was more effective at inducing mRNA expression than CpG or poly(I:C) in these cells (Fig. 3*B*, 3*C*). Bone marrow-derived macrophages (BMDMs) and TEPMs expressed ≈10- and ≈500-fold lower constitutive SerpinB2 mRNA levels than RPMs, respectively. After stimulation with LPS, BMDMs and TEPMs had ≈5- and ≈100-fold lower SerpinB2 mRNA than RPMs, respectively (data not shown). Constitutive SerpinB2 protein expression was barely detectable in BMDMs and TEPMs. After LPS stimulation, SerpinB2 protein levels remained barely detectable in BMDMs but were significantly induced in TEPMs, although levels were at least 2-fold lower than those seen in LPS-stimulated RPMs (data not shown).

LPS, but not CpG and poly(I:C), stimulation of CD11c$^+$ splenic DCs resulted in an ≈7-fold increase in SerpinB2 mRNA levels after 24 h (Fig. 3*D*). However, these levels were ≈10,000-fold lower than peak levels seen in LPS-activated RPMs at 6 h (note scale on the *y*-axis) (Fig. 3*D*). CD11c^{hi} conventional DCs (cDCs),

rather than CD11c^{+},120G8/CD317^{+} plasmacytoid DCs (pDCs), were responsible for this SerpinB2 mRNA expression, with LPS able to induce SerpinB2 expression in these cells both in vitro and in vivo (Fig. 3D). We were, however, unable to detect SerpinB2 protein expression in splenic cDCs or bone marrow-derived DCs by Western blotting.

To determine whether B cells or T cells express SerpinB2, splenocytes from SerpinB2^{+/+} mice were stimulated with LPS (100 ng/ml) or PHA (10 μg/ml) and IL-2 (10 IU/ml), respectively, for 6 and 24 h. CD14^{−}, CD19^{+} B cells and F4/80^{−},CD3^{+} T cells were sorted by FACS and analyzed by real-time RT-PCR. Although abundant SerpinB2 mRNA and protein were detected in the CD14^{+} or F4/80^{+} fractions, no mRNA expression was detected in the B or T cell fractions (data not shown). NK cells FACS sorted (NK1.1^{+}, CD3^{−}, F4/80^{−}, and CD11c^{−}) from spleen 18 h after OVA/CFA vaccination (44) also showed no detectable expression of SerpinB2 mRNA (data not shown).

The data demonstrated that macrophages are the main producers of SerpinB2 and that cDC also express low levels of SerpinB2 mRNA.

Splenic and bone marrow-derived DCs from SerpinB2^{−/−} mice show no changes in numbers, development, survival, and activity

A series of investigations were undertaken to determine whether a loss of SerpinB2 expression affects splenic or bone marrow-derived DCs. Splenic DC populations in SerpinB2^{−/−} mice were no different with respect to the numbers of cDCs, pDCs, or CD8/4[+] DCs and the ability of cDCs to induce allogeneic T cell proliferation (Supplemental Fig. 1). Generation of bone marrow-derived DCs from SerpinB2^{−/−} and SerpinB2^{+/+} mice in vitro using standard GM-CSF/IL-4 protocols also resulted in similar proliferative responses, frequencies, and levels of CD11c^{+} staining (data not shown). Furthermore, the cytokine responses and survival of these cells derived from SerpinB2^{−/−} and SerpinB2^{+/+} mice was not significantly different after anti-CD40/IFN-γ or LPS treatment (data not shown). These data suggest SerpinB2 deficiency does not result in overt differences in these DC populations.

SerpinB2^{−/−} macrophages are sufficient to promote Th1 immunity in vivo and in vitro

Macrophages are also well-known to influence Th1 responses (48), and they express high levels of SerpinB2. We thus sought to determine whether differences in macrophages from SerpinB2^{−/−} and SerpinB2^{+/+} might be responsible for the enhanced Th1 responses. Of the macrophage populations tested, RPMs express the highest constitutive and inducible levels of SerpinB2. OVA-pulsed, LPS-stimulated RPMs from SerpinB2^{−/−} and littermate control mice were therefore adoptively transferred into C57BL/6 mice. RPMs were used because these cells consistently express high levels of SerpinB2 protein (Fig. 3B, 3C) and represent fully differentiated F4/80^{hi} tissue macrophages that could be readily generated with >93% purity with no detectable F4/80^{−}CD11c^{+} DC contamination. Adoptive i.v. transfer of OVA and LPS-treated SerpinB2^{−/−} RPMs resulted in the induction of >4-fold more OVA-specific IFN-γ–secreting T cells than after transfer of similarly treated RPMs from SerpinB2^{+/+} mice (Fig. 4A).

To determine whether this phenomenon could be recapitulated in vitro, splenocytes from SerpinB2^{−/−} and littermate control mice were treated in vitro with anti-CD3. Splenocytes from SerpinB2^{−/−} mice showed significantly more IFN-γ–secreting cells than splenocytes from littermate control mice (Fig. 4B). In addition, when purified littermate T cells were cocultured with SerpinB2^{−/−} or SerpinB2^{+/+} RPM in the presence of anti-CD3, the former combination significantly increased the number of T cells secreting IFN-γ (Fig. 4C).

These data illustrate that SerpinB2 deficiency in macrophages is sufficient to enhance IFN-γ production by wild-type T cells in vivo and in vitro. The role of macrophages in the Th1 bias seen in
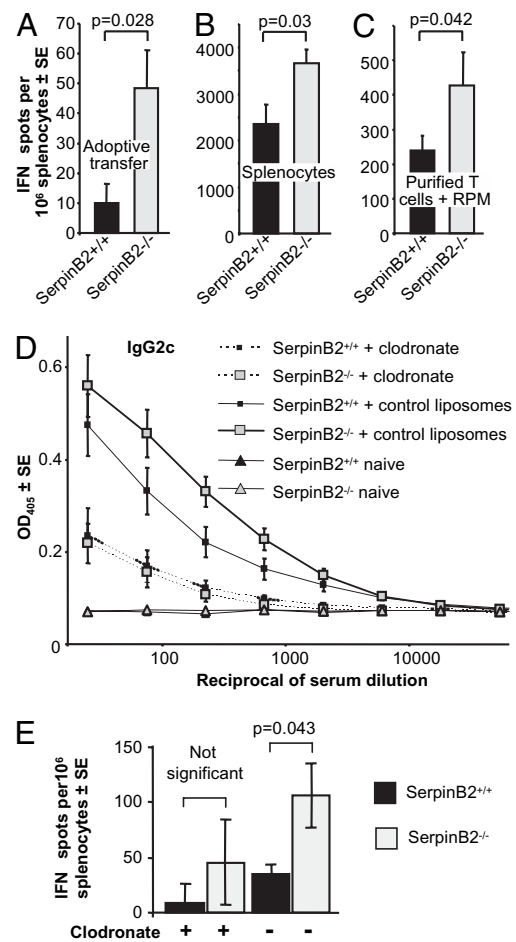

FIGURE 4. SerpinB2^{−/−} macrophages impart a Th1 bias in vivo and in vitro. *A*, OVA-sensitized and LPS-treated RPMs isolated from SerpinB2^{−/−} or SerpinB2^{+/+} (littermate) mice were adoptively transferred into C57BL/6 mice. After 10 d, splenocytes were analyzed by IFN-γ ELISPOT assay (n = 8/9 per group). Significance was calculated using the Mann-Whitney U test. *B*, Splenocytes (3 × 10^{4}/96 well in duplicate) from SerpinB2^{−/−} or SerpinB2^{+/+} (littermate) mice (n = 7/group) were treated with anti-CD3 Ab (0.5 μg/ml) for 48 h in an IFN-γ ELISPOT assay. Significance was calculated by *t* test assuming unequal variance. *C*, RPMs from SerpinB2^{−/−} or SerpinB2^{+/+} (littermate) mice (5 × 10^{4}/96 well) (n = 6/8 per group) were mixed in duplicate (1:1) with FACS-sorted (CD3^{+}) C57BL/6 T cells for 48 h in an IFNγ ELISPOT assay. The data were derived from two independent experiments, and significance was calculated by Kruskal-Wallis ANOVA, which contained a term for experiment. *D* and *E*, Mice were injected with liposomes containing clodronate to deplete macrophages or control liposomes. The next day, the mice were vaccinated with OVA/CFA and IgG2c ELISA (*D*) and ex vivo ELISPOT assays (*E*) undertaken (n = 6/group) as for Fig. 1.

SerpinB2^{−/−} mice was further supported by the observation that differences in IgG2c titres (Fig. 4D), and IFN-γ ELISPOT counts (Fig. 4E) between SerpinB2^{−/−} and SerpinB2^{+/+} mice were lost after depletion of macrophages with clodronate liposomes. The reduction in CFA-induced Th1 responses after clodronate liposome treatment has been observed previously (43).

SerpinB2^{−/−} RPM secrete more Th1-promoting cytokines after anti-CD40/IFN-γ stimulation in vitro

To gain more insight into how SerpinB2 deficiency might cause the increased T cell IFN-γ production seen in Fig. 4C, proinflammatory mediator levels were analyzed in the cell culture supernatants from this experiment. The increased IFN-γ secretion (Fig. 4C) was associated with significant increases in TNFα, IL-6, and MCP-1 but not IL-10 (Fig. 5A). IL-12 was not detected (data not shown).
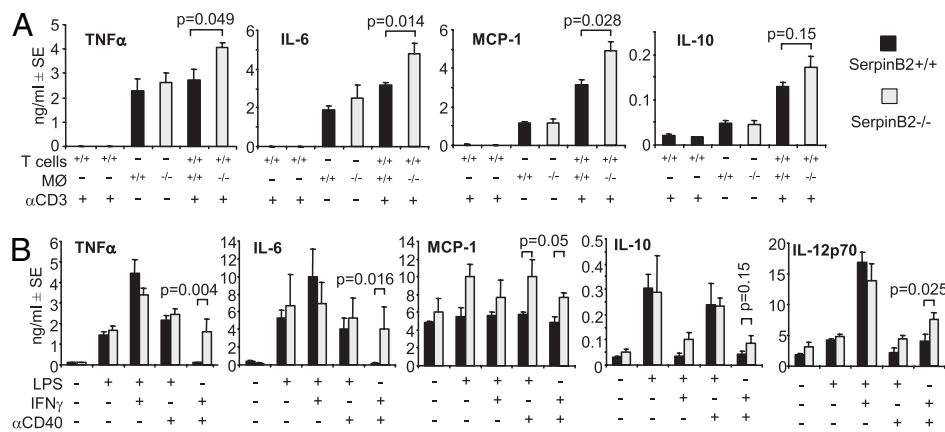
FIGURE 5. Macrophages from SerpinB2$^{-/-}$ mice secrete more Th1-promoting cytokines. *A*, The supernatants from the ELISPOT experiment described in Fig. 4*C* where analyzed for the indicated cytokines/chemokine by cytokine bead array. T cells were derived from SerpinB2$^{+/+}$ (+/+) mice or not added (−). RPMs were derived from SerpinB2$^{-/-}$ (−/−) or SerpinB2$^{+/+}$ (+/+) mice or not added (−). Anti-CD3 was added (+) or not (−). *B*, RPMs from SerpinB2$^{-/-}$ or SerpinB2$^{+/+}$ (littermate) mice (*n* = 6 per group) were treated with LPS, IFN-γ, and/or anti-CD40 Ab for 6 h. Supernatants were analyzed for the indicated cytokines/chemokine as above. Significances were calculated using *t* test or Mann-Whitney *U* test.

When purified RPMs (in the absence of T cells) were stimulated with LPS, IFN-γ, and/or anti-CD40, only a combination of anti-CD40 and IFN-γ induced the production of significantly more TNF-α, IL-6, and IL-12 from SerpinB2$^{-/-}$ when compared with SerpinB2$^{+/+}$ RPMs (Fig. 5*B*). Differences in MCP-1 levels approached significance under these conditions, and IL-10 levels were again not significantly different (Fig. 5*B*). Anti-CD40 or IFN-γ stimulation alone did not significantly induce cytokines in this assay (data not shown). CD40 expression levels were not significantly different for SerpinB2$^{-/-}$ and SerpinB2$^{+/+}$ RPMs, and no significant changes in survival between SerpinB2$^{-/-}$ and wild-type RPMs were observed in these experiments (data not shown).

Taken together, these data indicate that SerpinB2 expression in macrophages results in suppression of Th1-promoting cytokine secretion after interaction with T cells via IFN-γ and CD40. TNF-α, IL-6, and IL-12 have all been shown to promote Th1 responses, either indirectly and/or by acting directly on T cells (48–50).

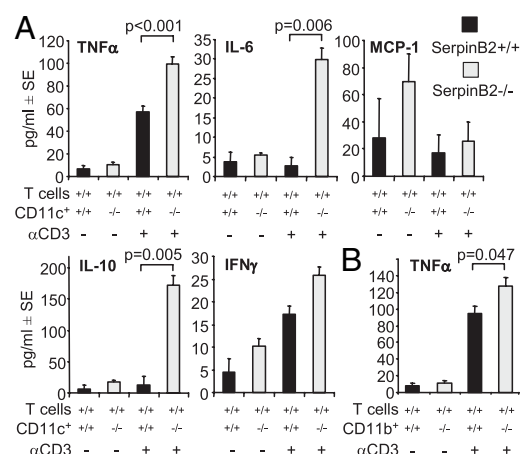*Increased Th1-promoting cytokines in draining lymph node APCs from SerpinB2$^{-/-}$ mice*

To determine whether increased Th1-promoting cytokine expression might be evident at the injection site in SerpinB2$^{-/-}$ mice, OVA/CFA vaccine injection sites from SerpinB2$^{-/-}$ and littermate control mice were analyzed by real-time RT-PCR. No increased levels of TNF-α, IL-6, and IL-12 p35 and p40 mRNA were detected in injection site tissue from SerpinB2$^{-/-}$ mice (data not shown). To determine whether differences in Th1-promoting cytokines might be apparent in draining lymph nodes, CD11c$^+$ and CD11b$^+$CD11c$^-$ cells were isolated from day 4 draining lymph nodes of vaccinated SerpinB2$^{-/-}$ and SerpinB2$^{+/+}$ mice and were cocultured with SerpinB2$^{+/+}$ T cells in the presence of anti-CD3. Cultures stimulated with SerpinB2$^{-/-}$ CD11c$^+$ cells produced significantly higher levels of TNF-α, IL-6, and IL-10 than cultures stimulated with SerpinB2$^{+/+}$CD11c$^+$ cells (Fig. 6*A*). No significant differences in IL-2, IL-4, IL-5, or IL-12 were seen in these cultures (data not shown). A similar experiment using SerpinB2$^{-/-}$CD11b$^+$ CD11c$^-$ cells also showed significantly increased TNF-α levels (Fig. 6*B*), but no significant changes in the other cytokines were observed in supernatants (data not shown).

Approximately 30% of CD11c$^+$ cells in the draining lymph node between 1 and 4 d after immunization expressed F4/80$^+$ (data not shown), consistent with previous reports (51). In an attempt to further define the cell types involved, CD11c$^+$ F4/80$^-$ and CD11c$^-$F4/80$^+$ cells were FACS sorted from draining lymph nodes and analyzed by

real-time RT-PCR. Both cell populations from SerpinB2$^{-/-}$ mice expressed significantly more TNF-α mRNA than littermate controls, and in wild-type mice, these populations also showed similar and low levels of SerpinB2 mRNA expression (data not shown). SerpinB2 deficiency thus appears to enhance Th1-promoting cytokine release in multiple myeloid APC populations in the draining lymph node.

Discussion

A role for SerpinB2 expression in the suppression of Th1 responses has hitherto not been reported and represents the first report of a physiological function associated with SerpinB2 expression in myeloid APCs. That this represents a true physiological role for SerpinB2 is supported by clinical findings in a series of inflammatory diseases associated with changes in SerpinB2 expression or sequence. SerpinB2 expression is reduced in pre-eclampsia (52, 53), and SerpinB2 polymorphisms have been associated with lupus (21, 22) and myocardial infarction (23), and these diseases have been associated with increased Th1 responses (17, 54–59). Furthermore, inflammatory diseases, such as asthma (18), scleroderma (60), and

FIGURE 6. Draining lymph node myeloid APCs secrete more Th1-promoting cytokines in SerpinB2$^{-/-}$ mice. *A*, CD11c$^+$ cells isolated from draining lymph nodes of SerpinB2$^{-/-}$ (−/−) and SerpinB2$^{+/+}$ (+/+) mice 4 d after OVA/CFA immunization were incubated for 72 h with SerpinB2$^{+/+}$ CD4$^+$ T cells (T cells $^{+/+}$) with (+) or without (−) anti-CD3 (0.5 µg/ml), and supernatants were analyzed for the indicated cytokines/chemokine. *B*, CD11b$^+$, CD11c$^-$ cells treated as for *A*.

periodontal disease (61, 62), have been associated with excessive SerpinB2 expression, and these diseases have been associated with increased Th2 or insufficient Th1 responses (18, 63, 64) (Supplemental Table 1). We therefore believe we have identified a physiologically and clinically relevant activity for inflammation-associated SerpinB2; SerpinB2 expression in myeloid APCs suppresses Th1 immunity by limiting Th1-promoting cytokine release after engagement with T cells.

SerpinB2-mediated inhibition of Th1-promoting cytokine release appears to require high levels of SerpinB2 expression. Wild-type RPMs expressed high levels of SerpinB2, and SerpinB2$^{-/-}$ RPMs secreted more Th1-promoting cytokines. Wild-type BMDMs expressed lower levels of SerpinB2, and SerpinB2$^{-/-}$ BMDMs did not secrete significantly increased levels of Th1 cytokines. High levels of SerpinB2 were expressed at the injection site in wild-type mice, and myeloid APCs from the draining lymph nodes in SerpinB2$^{-/-}$ mice secreted more Th1-promoting cytokines. SerpinB2 levels were not substantially elevated at the injection site in wild-type mice after vaccination with OVA formulated with poly(I:C) or CpG oligonucleotides, and no significant increases in IgG2c or IFN-γ responses were observed in SerpinB2$^{-/-}$ mice immunized with these vaccines (Supplemental Fig. 2). Splenic DCs or bone marrow-derived DCs from wild-type mice expressed low levels of SerpinB2, and these cells from SerpinB2$^{-/-}$ mice did not display a Th1-promoting phenotype.

The Th1-promoting phenotype was observed in multiple myeloid APC subsets in the draining lymph node of SerpinB2$^{-/-}$ mice, suggesting that SerpinB2 deficiency affects multiple lymph node APC subsets either directly or via paracrine effects. The relatively low SerpinB2 levels in the lymph node compared with the injection site suggests that high SerpinB2 expression at the injection site may program APCs that drain to the lymph node for reduced Th1-promoting cytokine release. Alternatively, a low-frequency APC subset in the lymph node may express high SerpinB2 levels and limit Th1 responses. However, T cell interaction with APCs is unlikely to result in SerpinB2 induction as SerpinB2 expression in RPMs and bone marrow-derived DCs was unchanged after anti-CD40/IFN-γ treatment (data not shown).

The molecular mechanism whereby SerpinB2 expression reduces Th1-promoting cytokine production remains elusive. The Th1 bias seen in PAI-1$^{-/-}$ mice (65, 66) might suggest a common mechanism involving uPA inhibition. However, we were unable to detect any changes in uPA activity in SerpinB2$^{-/-}$ mice, in agreement with previous studies (15). In addition, stimulating RPM with anti-CD40/IFN-γ in the presence of aprotinin, a potent inhibitor of plasmin, did not reverse the increase in Th1-promoting cytokines in SerpinB2$^{-/-}$ cells, suggesting uPA activation of plasmin during this assay is not involved (data not shown). Nevertheless, extracellular SerpinB2 is detectable in RPM culture supernatants (data not shown), consistent with previous reports (67). uPA receptor signaling may be involved, although work in cancer cells suggests that SerpinB2 and PAI-1 would have opposing signaling activities (10). Extracellular SerpinB2 may nevertheless influence uPA-dependent macrophage differentiation processes (26), resulting in cells that are programmed with a reduced ability to respond to T cell signals. SerpinB2 binding to one or more of the annexin family (29, 32) may be involved in the Th1 phenotype, but a SerpinB2-mediated inhibition of annexin A1 degradation (29) would be expected to result in an increase in Th1 responses (68). Alternatively, an intracellular activity of SerpinB2 is involved. In a large range of experiments, we were unable to find any evidence for SerpinB2 expression significantly influencing antiviral activity (6), IFN response factor 3 (33), IL-1β secretion (30), IL-18 secretion, retinoblastoma protein levels (2, 12, 31), or apoptosis (5, 14, 27) in macrophages (data not shown). However, we have identified enhanced alternative NF-κB signaling in SerpinB2$^{-/-}$ RPMs and have confirmed binding of SerpinB2 to the proteasomal subunit, proteasome subunit, β type 1 (35) (data not shown). The proteasome is intimately associated with CD40 and NF-κB signaling and macrophage function and differentiation. Resolving the molecular mechanism whereby SerpinB2 suppresses Th1 responses should lead to an improved understanding of the diseases associated with SerpinB2 dysregulation and polymorphisms and may open new avenues for treatment.

Disclosures
The authors have no financial conflicts of interest.

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