Published in final edited form as:

Arthritis Rheum. 2011 December ; 63(12): 3897–3907. doi:10.1002/art.30629.

Co-Opting Endogenous Immunoglobulin for the Regulation of Inflammation and Osteoclastogenesis in Humans and Mice

Lindsay M. MacLellan¹, Jennifer Montgomery¹, Fujimi Sugiyama¹, Susan M. Kitson¹, Katja Thümmeler¹, Gregg J. Silverman², Stephen A. Beers³, Robert J. B. Nibbs¹, Iain B. McInnes¹, and Carl S. Goodyear¹

¹University of Glasgow, Glasgow, UK

²University of California at San Diego, La Jolla

³Southampton University, Southampton, UK

Abstract

Objective—Cells of the monocytic lineage play fundamental roles in the regulation of health, ranging from the initiation and resolution of inflammation to bone homeostasis. In rheumatoid arthritis (RA), the inflamed synovium exhibits characteristic infiltration of macrophages along with local osteoclast maturation, which, together, drive chronic inflammation and downstream articular destruction. The aim of this study was to explore an entirely novel route of immunoglobulin-mediated regulation, involving simultaneous suppression of the inflammatory and erosive processes in the synovium.

Methods—Using in vivo and in vitro studies of human cells and a murine model of RA, the ability of staphylococcal protein A (SPA) to interact with and modulate cells of the monocytic lineage was tested. In addition, the efficacy of SPA as a therapeutic agent was evaluated in murine collagen-induced arthritis (CIA).

Results—SPA showed a capacity to appropriate circulating IgG, by generating small immunoglobulin complexes that interacted with monocytes, macrophages, and preosteoclasts. Formation of these complexes resulted in Fc γ receptor type I-dependent polarization of macrophages to a regulatory phenotype, rendering them unresponsive to activators such as interferon- γ . The antiinflammatory complexes also had the capacity to directly inhibit differentiation of preosteoclasts into osteoclasts in humans. Moreover, administration of SPA in the early stages of disease substantially alleviated the clinical and histologic erosive features of CIA in mice.

Conclusion—These findings demonstrate the overarching utility of immunoglobulin complexes for the prevention and treatment of inflammatory diseases. The results shed light on the interface between immunoglobulin complex-mediated pathways, osteoclastogenesis, and associated

© 2011, American College of Rheumatology

Address correspondence to Carl S. Goodyear, PhD, Institute of Infection, Immunity and Inflammation, College of Medicine, Veterinary Medicine and Life Sciences, University of Glasgow, Sir Graeme Davies Building, 120 University Place, Glasgow G12 8TA, UK. Carl.Goodyear@glasgow.ac.uk.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Goodyear had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Silverman, McInnes, Goodyear.

Acquisition of data. MacLellan, Montgomery, Sugiyama, Kitson, Thümmeler, Beers.

Analysis and interpretation of data. MacLellan, Montgomery, Nibbs, Goodyear.

pathologic processes. Thus, therapeutic agents designed to harness all of these properties may be an effective treatment for arthritis, by targeting both the innate inflammatory response and prodestructive pathways.

Monocytes differentiate into either macrophages or osteoclasts depending on their response to specific intra- and extracellular signals (1,2). These cells play pivotal roles in the generation of adaptive immune responses, initiation and resolution of inflammation, and bone homeostasis (3). In chronic inflammatory conditions such as rheumatoid arthritis (RA), these cells have crucial roles in perpetuating disease pathogenesis. Monocytes and macrophages are the primary source of the inflammatory cytokines and chemokines that drive the chronicity of the synovial lesion (4,5), while the increased differentiation of osteoclasts from cells present in the joint (6,7) is considered critical for the development of the major erosive lesion.

The local environment has a dramatic influence on macrophage maturation and can drive the development of a spectrum of functionally diverse phenotypes (8). Two of the functional extremes are the antiinflammatory “regulatory” macrophages and the proinflammatory “classically” activated macrophages. The regulatory phenotype is characterized by increased expression of interleukin-10 (IL-10) but decreased expression of IL-12. In contrast, the proinflammatory phenotype is defined as Th1-driven (interferon- γ [IFN γ]-primed) and characterized by increased expression of IL-12 and inducible nitric oxide synthase (8). Therapeutic strategies with the potential to polarize macrophages to a regulatory phenotype are of intense interest in the generation of new approaches to treat patients during the chronic self-perpetuation phase of RA (5).

One potential therapeutic approach for inflammatory diseases comes from an understanding of immune complexes (ICs) and their interactions with macrophages. It has been recognized for several decades that ICs play a central role in the pathogenesis of RA (9), being responsible for driving inflammation and, indirectly, joint erosion, via Fc γ receptor (Fc γ R)-mediated interactions (10). In the right context, ICs can have antiinflammatory properties (8), and this is partly due to their ability to contribute to the generation of regulatory macrophages (11). Recent studies have demonstrated that small, preformed ICs (12), intravenous immunoglobulin (IVIG) complexes (13,14), and antibodies to soluble serum proteins (15) can be used to ameliorate arthritis in passive transfer autoimmune models such as K/BxN serum-induced inflammatory arthritis. However, such unoptimized therapies as IVIG have little clinical benefit in RA.

Staphylococcal protein A (SPA), a microbial protein widely used for therapeutic antibody purification and formerly used as a column-bound apheresis therapy for severe RA (16), has the ability to co-opt circulating IgG and exclusively form small, defined hexameric complexes, or (IgG₂SPA)₂ (17–19). Exploiting the ability of SPA to hijack endogenous IgG may facilitate a novel macrophage-targeting approach, aimed at driving Fc γ R-mediated signaling toward regulatory pathways that can modify autoimmune inflammatory diseases.

In this study, we show that SPA-IgG immune complexes (SICs) have the ability to ameliorate antigen-induced arthritis, by inhibiting both the clinical and pathologic aspects of murine collagen-induced arthritis (CIA). Mechanistically, SICs modulate macrophage responsiveness to proinflammatory cytokines via Fc γ R, and thus alter the phenotype of these cells. Furthermore, we show, for the first time, that specific ICs can interact with preosteoclasts, can dramatically inhibit osteoclastogenesis, and can substantially reduce osteoclast abundance in the joint. We conclude that coopting endogenous IgG into ICs may provide an alternative therapy for RA and other autoimmune/inflammatory diseases, by targeting both inflammatory and erosive aspects of the disease process.

MATERIALS AND METHODS

Mice

DBA/1J and C57BL/6 mice (ages 7–10 weeks) were purchased from Charles River. Mice of the μ MT strain were bred and maintained at the University of Glasgow, UK. $\text{Fc}\gamma\text{R}^{-/-}$, $\text{Fc}\gamma\text{RI}^{-/-}$, and $\text{Fc}\gamma\text{RII}^{-/-}$ mice (C57BL/6 background) were bred and maintained at the University of Southampton, UK. The Ethical Review Process Committee and the UK Home Office approved all experimental procedures.

In vivo tracking of SPA

Recombinant SPA (rSPA; Repligen), or ovalbumin (OVA) as a control, was conjugated to Alexa Fluor 488–labeled *N*-hydroxysuccinimide (Molecular Probes), as previously described (20). In μ MT mice, 6 mg of mouse IgG (Jackson Immunochemicals) in phosphate buffered saline (PBS) was injected intravenously. Labeled protein (300–500 μ g of SPA-488 or OVA-488) was then injected intraperitoneally (IP), and 2 hours later, the blood and spleens were harvested and erythrocyte-free single cell suspensions were prepared.

Flow cytometry

Cells were preincubated with or without Fc Block and stained with specific antibodies or isotype controls. Data were acquired on a FACSCaliber LSRII (Becton-Dickinson) or MACSQuant (Miltenyi) flow cytometer, and results were analyzed with FlowJo software (Tree Star).

Generation of immune complexes

To generate the SICs, rSPA (SPA-488) at a concentration of 37.5 μ M was mixed with 150 μ M human or mouse IgG in PBS at 37°C for 1 hour. To generate the OVA plus polyclonal IgG (OpIg) control, 37.5 μ M of OVA (OVA-488) was mixed with 150 μ M of mouse IgG in PBS at 37°C for 1 hour. Prior to being used in these experiments, the IgG was centrifuged at 13,000 revolutions per minute for 5 minutes, to remove large IgG complexes.

Generation and stimulation of murine bone marrow–derived macrophages (BMMs) and preosteoclasts

Cells were flushed from the mouse femurs and tibiae. In some studies, monocytes were enriched by magnetic negative selection (StemCell Technologies). Bone marrow monocytes or enriched monocytes were cultured in complete RPMI with 20% L929-conditioned medium or 30 ng/ml recombinant macrophage colony-stimulating factor (M-CSF) for 5–6 days to generate BMMs, or with 30 ng/ml M-CSF and 50 ng/ml RANKL for 5 days to generate preosteoclasts. On days 5–6, the BMMs and preosteoclasts were removed and either used directly in flow cytometry or washed, reseeded, and left to adhere overnight prior to stimulation.

BMMs were stimulated for 6–24 hours with lipopolysaccharide (LPS) (100 ng/ml *Escherichia coli* O127:B8; Sigma-Aldrich) in the presence or absence of SICs, SPA, or IgG. In some instances, BMMs were prestimulated overnight with IFN γ (100 units/ml) prior to these other treatments. To analyze the macrophage response to OVA ICs (designated M Φ -II in Figure 3), after prestimulation of the BMMs with IFN γ , the cells were treated with LPS and 150 μ g/ml ICs consisting of OVA and rabbit anti-OVA IgG, as previously described (21).

Enzyme-linked immunosorbent assay (ELISA)

Cytokine and anticollagen antibody levels were assayed by ELISA, using appropriately diluted sera or culture supernatants. Mouse IL-10 and IL-12p40 (BD Biosciences), and human IL-10, IL-12, and tumor necrosis factor α (TNF α ; Biosource) were assayed in accordance with the manufacturers' instructions. Anti-type II collagen (anti-CII) antibody titers in individual sera were evaluated using ELISA-grade collagen (Chondrex) and detected with horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 or IgG2a (Southern Biotechnology). Total IgG was determined using an unlabeled anti-mouse IgG capture antibody and detected with HRP-conjugated anti-mouse IgG1 or IgG2a. Antibody ELISAs were developed using OPD substrate (Sigma).

Induction and assessment of arthritis

CIA was induced in DBA/1J mice with 100 μ g of bovine CII emulsified in Freund's complete adjuvant (MD Biosciences) on day 0, and boosted on day 21 with an IP injection of CII in PBS (22). Starting on day 21, mice were scored by a treatment-blinded researcher (LMM) for clinical signs of arthritis, as previously described (23). On day 25, mice were randomized to receive an IP injection of 100 μ g rSPA or vehicle control (pyrogen-free PBS). Treatment was continued every other day.

The hind paws were histologically scored by treatment-blinded researchers (LMM and CSG) for inflammation and joint damage (24), in which 0 = healthy, 1 = mild, 2 = moderate, and 3 = severe. The researchers scored 3 sections per knee, and the mean score per treatment group was calculated.

In addition, the hind paws were assessed by immunohistochemistry for the presence of osteoclasts. Sections were deparaffinized, and antigen retrieval was performed. Slides were blocked in 3% horse serum/Tris buffered saline and stained with a rabbit polyclonal IgG against cathepsin K (ProteinTech Group) or normal rabbit IgG. Slides were developed using the ImmPRESS Anti-Rabbit Ig (peroxidase) substrate kit (Vector), and counterstained with hematoxylin. The bone surface area and the numbers of cathepsin K-positive cells per section were determined for each knee joint, and the number of cells per mm² of bone was calculated.

Restimulation of lymph node (LN) cells

Single cell suspensions were prepared from inguinal LNs. Cells were cultured in triplicate in 96-well plates at 6×10^5 cells/well in complete RPMI. Cells were restimulated with medium, 30 μ g/ml CB11 peptide (optimal concentration; Chondrex), or 30 μ g/ml of the irrelevant peptide MOG_{35–55}. Proliferation was analyzed 88 hours thereafter by assessing ³H-thymidine incorporation (GE Healthcare) for the last 16 hours of culture.

Real-time quantitative polymerase chain reaction (PCR)

Cell and tissue RNA was isolated using Qiagen Mini and Micro kits in accordance with the manufacturer's instructions. Tissue samples were disrupted in liquid nitrogen using a pestle and mortar. To quantify RNA transcripts, complementary DNA (cDNA) was prepared using the Stratagene Affinity Script Multiple Temperature cDNA Synthesis Kit. Real-time quantitative PCR using SYBR Green (Applied Biosystems) was carried out using the Applied Biosystems 7900HT Fast Real-Time PCR System. Specific transcript levels were normalized to those for GAPDH, and the $\Delta\Delta C_t$ calculation method was used to determine gene expression, as previously described (25).

Generation and stimulation of human peripheral blood monocyte–derived macrophages

Buffy coats of healthy human blood were obtained from the Scottish Blood Transfusion Service, and their use was approved by the Glasgow East Ethics Committee. Peripheral blood mononuclear cells were separated on Histopaque (Sigma-Aldrich), and monocytes were purified using the CD14-positive selection EasySep kit (StemCell Technologies). The monocytes were cultured in complete RPMI with 10 ng/ml human M-CSF (PeproTech) for 7 days in the presence or absence of SICs, SPA, or IgG. On day 7, cultures were stimulated with LPS (100 ng/ml) and supernatants were harvested for analysis of cytokine content. For the investigation of IFN γ -mediated signaling, monocytes were cultured for 6 days with M-CSF, washed, and treated with either SICs, SPA, or IgG for 48 hours. These cultures were then stimulated with 10 units/ml of IFN γ (PeproTech) for 10 minutes and immediately used for immunoblotting or imaging.

Immunoblotting

Whole cell extracts were obtained by lysing cells in radioimmunoprecipitation assay extraction buffer with protease and phosphatase inhibitors. Protein amounts were quantified with the BCA Protein Assay (Pierce), according to the manufacturer's instructions. Twenty micrograms of cell lysate was fractionated on 4–12% Bis-Tris Gels, transferred to PVDF membranes (Invitrogen), and incubated with antibodies specific for phosphorylated STAT-1 tyrosine 701, total STAT-1, or β -actin (Abcam). Membranes were washed and incubated with HRP-labeled anti-IgG antibodies and developed with SuperSignal West Pico substrate (Pierce).

Immunofluorescence imaging

Chamber slide cultures were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Phosphorylated STAT-1 was visualized in cells by permeabilizing with 90% ethanol in PBS and staining with a biotinylated anti-phosphorylated STAT-1 antibody. This was followed by staining with streptavidin–HRP, biotinylated tyramide (Invitrogen), and, finally, streptavidin–Alexa Fluor 647. The sections were also stained with DAPI and mounted in Vectashield (Vector). Fluorescence imaging was conducted using an LSC fluorescence microscope (Compucyte) with a Hamamatsu Orca ER digital camera and Openlab digital imaging program (Improvision).

Generation of human peripheral blood monocyte–derived osteoclasts

Monocytes (1×10^6) were cultured in α -minimum essential medium (10% fetal bovine serum, 2 mM L-glutamine, 100 μ g/ml penicillin, 100 μ g/ml streptomycin) with 30 ng/ml M-CSF and 100 ng/ml RANKL (PeproTech) in the presence or absence of SICs, SPA, OpIg, or IgG. The medium was changed every 3 days, and on day 7, the cultures were evaluated for osteoclast differentiation. Slides were stained for tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (Sigma-Aldrich). Osteoclasts were identified under light microscopy by the presence of ≥ 3 nuclei (identified as purple staining); osteoclast precursor cells were identified as TRAP-positive cells with 1 or 2 nuclei.

Statistical analysis

GraphPad Prism was used for all statistical analyses: *t*-tests, Mann-Whitney U tests, one-way analysis of variance (ANOVA) with post hoc tests, or two-way ANOVA with repeated measures, as appropriate. Data are expressed as the mean $\pm$ SD, except for clinical scores of CIA, which are given as the mean $\pm$ SEM. *P* values less than or equal to 0.05 were considered significant, and all tests were 2-sided.

RESULTS

Binding of SPA-IgG complexes to monocytes, macrophages, and preosteoclasts via FcγRI

SPA binds to B cells in a B cell receptor (BCR)-dependent manner. However, it can also interact with non-B cell and non-T cell populations in vivo (20). We analyzed the non-B cell binding of fluorescently labeled SPA in vivo. In the blood of mice injected with SPA-488, we observed considerable binding to monocytes but minimal binding to neutrophils (Figures 1A and B). SPA-488 also interacted with differentiated monocytes, i.e., CD11b⁺ macrophages, in the spleen (Figure 1C).

In B cell/immunoglobulin-deficient μMT mice, SPA was able to interact with circulating monocytes only if the mice had been reconstituted with IgG (Figure 1D). To further investigate the interaction of SPA and IgG with monocytes, macrophages, or preosteoclasts, we generated SPA-488-IgG immune complexes (SIC-488) (data not shown) at a molar ratio that is consistent with what would be formed in the circulation when excess IgG, or (IgG₂SPA)₂ (17–19), is present. SIC-488 showed significant binding both to BMMs and to preosteoclasts, while SPA-488, OVA-488 (data not shown), or OpIg-488 were unable to bind to these cells (Figure 2).

As was indicated in Figure 1, SPA-488 showed substantial binding only to monocytes. Importantly, whereas both monocytes and neutrophils express FcγRIIb, FcγRIII, and FcγRIV, only monocytes express FcγRI (26) (data not shown). Thus, as anticipated, SIC-488 was unable to bind to BMMs or to preosteoclasts that were deficient in their expression of FcγRI (Figure 2).

Skewing of macrophages to a regulatory phenotype by SICs

The aforementioned findings and those from previous studies using experimental ICs (refs. 21, 27, and data not shown) raised the possibility that SICs would interact with, and polarize, macrophages toward a regulatory phenotype. The addition of SICs, and not SPA or IgG alone, in the absence of a Toll-like receptor 4 agonist (LPS) was able to induce significant production of IL-10 (Figure 3A and data not shown), a phenomenon not noted previously with other experimental ICs (21,27). Moreover, the addition of SICs along with stimulation with LPS resulted in a significant inhibition of IL-12 secretion (Figure 3A). Levels of TNFα and nitric oxide were unchanged, and transforming growth factor β was not detectable (data not shown). The induction of IL-10 was also confirmed at a transcriptional level, in which SICs induced an 8-fold increase in IL-10 transcripts (data not shown). Crucially, mannose receptor expression did not change (data not shown), suggesting that SPA does not induce a formal regulatory phenotype.

To further define this apparently novel regulatory macrophage lineage, BMMs from DBA/1J and C57BL/6 mice were primed with IFNγ, and 16 hours later, the cells were stimulated with LPS in the presence or absence of SICs. As a result, upon stimulation of the cells with SICs and LPS, the levels of CD86 were up-regulated, which was also observed with SICs alone (data not shown), whereas there was an abrogation of the up-regulated expression of class II major histocompatibility complex (MHC) (Figures 3B and C and data not shown). This is distinct from the observations made with other experimental ICs, which, in general, upregulated class II MHC and CD86 (Figures 3B and C) (21,27). In addition, neither LIGHT nor CCL-1 transcript levels, both of which are known to be associated with the regulatory phenotype, were altered by stimulation with SICs plus LPS when compared to LPS alone (data not shown).

Polarization of macrophages by SICs via FcγRI interactions

Studies on the mechanisms of experimental ICs have demonstrated that these complexes impart their effects through ligation of FcγRI (11). Based on these studies and our own findings (as shown in Figure 2), we hypothesized that SICs would interact with macrophages in a similar way. To determine whether FcγRI or an alternative Fc receptor was the main mechanism of interaction, we generated BMMs from C57BL/6, FcRγ^{-/-}, FcγRI^{-/-}, and FcγRII^{-/-} mice. When BMMs from C57BL/6 mice were treated with SICs, regulatory macrophages were generated (Figure 3D). However, in the absence of either the FcRγ chain, i.e., lack of the Fc receptors FcγRI, FcγRIII, and FcγRIV (data not shown), or in the absence of FcγRI alone (Figure 3D), SICs were unable to modify the effect of LPS and skew BMMs toward a regulatory phenotype. Interestingly, SICs were still effective at inducing regulatory macrophages in the absence of FcγRII (data not shown).

Amelioration of murine arthritis by treatment with SPA

Given the ability of SICs to interact with monocytes, macrophages, preosteoclasts, and B cells, and, more specifically, their ability to polarize macrophages to a regulatory phenotype, we investigated the potential of treatment with SPA to ameliorate disease in the early phase of inflammatory arthritis. Treatment of mice with SPA significantly decreased the severity and incidence of disease (Figures 4A and B). Histomorphometric evaluation revealed that, compared with vehicle-treated mice, SPA-treated mice had significantly fewer features of inflammatory disease and displayed markedly less hyperplasia, infiltration, and cartilage and bone erosion (Figures 4C–E).

In addition, the evaluation of osteoclast numbers in the joints illustrated that SPA-treated mice had substantially fewer osteoclasts present in the joints (Figures 4F–H). This latter finding could arise from a suppression of macrophages or a reduction in inflammation, which thus would decrease RANKL, IL-1, or M-CSF release, or alternatively could reflect a direct, and unidentified, effect of SPA/SICs upon preosteoclasts and the resulting osteoclastogenesis.

Skewed anti-CII antibody response and increased serum IL-10 levels following SPA treatment

In vivo administration of SPA results in the depletion of V_H3 BCR-encoded B cells (28). In the murine CIA model, this depletion occurs (data not shown); however, susceptible B cells only represent ~3% of the repertoire. Total B cell depletion late in CIA has no effect on clinical disease (29), and although we cannot conclusively rule out the impact of this small level of depletion, it is unlikely to be the main contributing factor to such a dramatic clinical effect.

To identify alternative factors for the clinical effect of SPA, we investigated the established adaptive immune response. Surprisingly, treatment with SPA resulted in increased ex vivo lymphocyte proliferative responses to collagen peptide (Figure 5A). Moreover, the levels of CII-specific IgG2a antibodies were unaffected, whereas anti-CII IgG1 titers were elevated by SPA treatment (Figures 5B and C), thus demonstrating that in SPA-treated mice, the capacity to generate and maintain a class-switched antibody response with T cell help, albeit skewed to the IgG1 subclass, is retained. In addition, we also observed a significant increase in serum IL-10 levels with SPA treatment (Figure 5D).

Activation of macrophages with skewing to a regulatory phenotype can lead to an increase in IgG1 antibodies and an increase in the secretion of IL-10 (30), and SPA, in the form of SICs, can lead to a regulatory macrophage phenotype (Figure 3). Therefore, we considered

the possibility that SPA-mediated disease suppression may occur via the modulation of macrophage function.

Alteration of macrophage responsiveness to IFN γ by SPA

Although IFN $\gamma^{-/-}$ mice have exacerbated disease in the CIA model, treatment with IFN γ in the early stages of disease can exacerbate the clinical features even further (31). To address the capacity of SPA to alter macrophages in vivo, mice with CIA were treated with SPA or vehicle control, and on day 42, the mice received an IP injection of IFN γ . Three hours later, peritoneal macrophages were harvested and RNA was extracted for gene expression analyses. As expected, the addition of IFN γ in the vehicle treatment group resulted in a significant increase in the expression of the IFN γ -inducible genes IP10 and IRF1. In contrast, macrophages from the SPA-treated mice were completely unresponsive to IFN γ (Figure 5E).

Inhibition of inflammatory cytokine production and reduction in responsiveness to IFN γ by SICs in human macrophages

To confirm that SIC modulation was not a species-specific phenomenon, we investigated whether human monocyte polarization could be affected by SIC treatment. Human blood monocyte-derived macrophages were treated with SICs and stimulated with LPS. The addition of SICs resulted in a 46% decrease in the secretion of IL-12 ($P < 0.05$), and the levels of IL-10 were undetectable in the cultures (data not shown). Unlike in mouse BMMs (data not shown), SICs were able to decrease the production of TNF α in human macrophages by 76% ($P < 0.001$) (Figure 6A).

To extend our studies to demonstrate that SICs can also impair the capacity of proinflammatory cytokines to “classically” activate human macrophages, we cultured human blood-derived macrophages with SPA, IgG, or SICs for 48 hours prior to stimulation with IFN γ . The addition of either SPA or IgG alone was unable to inhibit the ability of IFN γ to activate STAT-1 (Figures 6B and C). The presence of SICs, however, considerably inhibited the phosphorylation of STAT-1 in response to IFN γ (Figures 6B–D).

Inhibition of osteoclastogenesis by SICs

SICs can interact with preosteoclasts (as shown in Figure 2) and these cells express Fc γ RI (data not shown). Furthermore, in vivo treatment is associated with a decrease in osteoclast abundance in the mouse joint (Figures 4F and H). To investigate whether SICs can alter the differentiation of preosteoclasts, human CD14 $^{+}$ monocytes were cultured in the presence of RANKL and M-CSF. The addition of SICs on day 1 resulted in the downregulation of CD115 (c-Fms), the M-CSF receptor, 6 hours posttreatment (Figure 6E).

To investigate whether this decrease in the level of CD115 was associated with a decrease in the subsequent ability of preosteoclasts to differentiate into osteoclasts, the number of multinucleated (≥ 3 nuclei) TRAP $^{+}$ cells was assessed on day 7 (Figures 6F and G). We observed a near complete inhibition of human osteoclast differentiation. Commensurate with this, there was no significant difference in the total number of viable cells in the cultures on day 7 (data not shown). Crucially, delaying the addition of SICs until day 5 (a time firmly associated with the initiation of osteoclast differentiation and emergence of cells capable of bone resorption [32,33]), led to the suppression of cell maturation (data not shown).

DISCUSSION

In this study, we evaluated the ability of SPA to ameliorate inflammatory arthritis in the early stages of disease and investigated the underlying mechanism of action. Although it

remains to be determined whether this form of therapy is efficacious in established disease, the results of our study show that SPA is a strong inhibitor of the inflammatory and erosive aspects of CIA. We show that these effects are due to the formation of immunoglobulin complexes, which are able to engage Fc γ RI, polarizing macrophages to a regulatory phenotype. We further demonstrate that SICs are a potent inhibitor of IFN γ -mediated macrophage activation, and we predict that this occurs via Fc γ RIII-mediated suppression of IFN γ RII expression (13). Furthermore, SICs can interact with preosteoclasts and inhibit their differentiation into mature osteoclasts.

SPA binds the Fc and variable heavy chains (V_H3) of immunoglobulin (28). These interactions do not inhibit the ability of IgG to engage Fc receptors (34). The ability of SPA to interact with the V_H3 BCR expressed on B cells leads to the induction of apoptosis (20,35–38) via an Fc γ RIII-dependent mechanism (Goodyear CS and Silverman GJ: unpublished data). In addition, SPA was the active component of a discontinued US Food and Drug Administration–approved apheresis therapy for inflammatory and autoimmune diseases, including severe RA (39). There has been longstanding controversy over the mechanism of action and unpredictable efficacy of the therapy for inflammatory and autoimmune diseases. Several hypotheses have been proposed, including removal of immunoglobulin and/or ICs, modification of small circulating ICs, and B cell depletion (40). A salient finding was that during each treatment, SPA could be proteolytically cleaved and enter the patient's circulation (41). The quantity of SPA entering was significantly less than the amount of circulating immunoglobulin, and Hanson and colleagues demonstrated that, when excess immunoglobulin is present, SPA will form only one type of immunoglobulin complex (17,42). Our findings (as shown in Figures 1 and 2) show that these homogeneous complexes can interact with monocytes, macrophages, and preosteoclasts via an Fc γ RI-mediated interaction. It is interesting to speculate that the administration of SPA would be of greater benefit than the suboptimal administration via uncontrollable proteolytic cleavage.

To put the potential of SICs in some perspective, several studies have shown that ICs present in IVIG (13,14) or generated in alternative ways (12,15) have the ability to inhibit inflammatory responses. Unlike our findings with SPA (Figure 4), these ICs have never been shown to be efficacious in active-immunization murine models of RA or in patients with RA. The formation of ICs in IVIG is a heterogeneous process, due to the diverse binding interactions, and is therefore nonstandardized for size and content, suggesting that IVIG is far from an optimized therapy. An alternative way to emulate IVIG ICs is the use of antibodies to immunologically inert serum protein, which can ameliorate arthritis in passive transfer autoimmune models. Unlike the findings in the present study (Figure 3), these complexes are dependent on the presence of Fc γ RIIb (15).

It is interesting to speculate that these differences may be attributable to the nature of the immunoglobulin used. The majority of previous studies have utilized xenogenic immunoglobulin, primarily rabbit IgG, to generate ICs (15,43). The interaction of rabbit IgG with murine Fc receptors has never been specifically investigated; however, results would suggest that rabbit IgG has a certain order-of-magnitude lower affinity for mouse Fc receptors compared to mouse IgG (44–47). It is feasible to envisage that rabbit IgG ICs will have altered binding activity to mouse Fc receptors that could result in dramatically different outcomes when compared to mouse IgG ICs. In this study, with the use of SPA, we were able to directly overcome any xenogenic-mediated differences and generate optimal homogeneous complexes with endogenous IgG, and it is this entity that contains immunomodulatory potential.

The alteration of monocytes and macrophages by ICs has been studied intensively, demonstrating that macrophages stimulated with ICs can be polarized to a regulatory

phenotype (21). Macrophage plasticity, a recently described phenomenon (8), would suggest that phenotypes are not definitive but part of a spectrum. Our data suggest that SIC-stimulated macrophages are in the regulatory area of the spectrum and may have the capacity to regulate the inflammatory response by being unresponsive to inflammatory cytokines (Figures 5 and 6), producing antiinflammatory cytokines (i.e., IL-10 in Figure 3) and by altered antigen presentation via class II MHC (Figure 3 and currently under investigation). We postulate that the difference with our SICs when compared to experimental ICs in previous studies is based on the affinity of autogenic IgG to Fc receptors compared to that of xenogenic IgG.

Monocyte-derived osteoclastogenesis is considered critical to the erosive RA process (48), and it has been suggested that in RA, circulating monocytes are primed prior to their arrival in the joint. Maturation of these monocytes is driven by M-CSF, via CD115, and RANKL (2). In juxtaposition to earlier studies illustrating the induction of bone loss and osteoclast accumulation via heat-aggregated IgG (49), our findings demonstrate that SICs not only are capable of directly interacting with monocytes and preosteoclasts via an Fc γ RI-mediated interaction (Figure 2), but also have the ability to down-regulate CD115 and inhibit osteoclastogenesis (Figure 6). The mechanisms underlying this SIC-mediated inhibition of osteoclastogenesis remain to be determined (currently under investigation). However, it is interesting to speculate that the interaction of SICs with Fc receptors on circulating monocytes, or even those that have entered the joint, might alter the threshold for initiation of M-CSF and RANKL signaling, thereby inhibiting the ability of monocytes and preosteoclasts to mature into fully functional osteoclasts.

In conclusion, monocyte/macrophage-targeting therapeutic approaches offer a rich potential for the treatment of human inflammatory diseases. We herein provide a novel approach that utilizes the ability of SPA to co-opt endogenous IgG, forming highly regulated complexes with defined size and shape (17,18). SPA, which presumably arose in *Staphylococcus aureus* to interfere with the host inflammatory responses required for immunogenicity of microbial antigens, can also serve as a potent inhibitor of autoimmune inflammatory disease. The antiinflammatory mode of action appears to operate via the modification of macrophage responsiveness to inflammatory signals and the generation of a regulatory macrophage that is characterized by IL-10 production but without the conventional regulatory phenotype. Finally, we provide a hitherto unrecognized effect of ICs on osteoclastogenesis, providing a second potent pathway whereby defined ICs could mediate therapeutic benefit.

Acknowledgments

We thank J. Reilly for technical assistance and Drs. M. J. Glennie, S. Milling, H. J. Willison, and C. Linington for helpful comments on the manuscript.

Supported by funding from the University of Glasgow, Arthritis Research UK, and Medical Research Scotland (Vipiana Award) (to Dr. Goodyear) and by a Capacity Building Award in Integrative Mammalian Biology (to Ms Montgomery and Dr. Goodyear) funded by the Biotechnology and Biological Sciences Research Council, British Pharmacological Society Integrative Pharmacology Fund (donors AstraZeneca, GlaxoSmithKline, and Pfizer), Knowledge Transfer Network, the Medical Research Council, and the Scottish Further and Higher Education Funding Council. Dr. Thümmel's work was supported by the DFG (postdoctoral fellowship TH1599/1-1). Dr. Goodyear's work was also supported by a nonclinical career development fellowship from Arthritis Research UK (grant 17653).

REFERENCES

1. Geissmann F, Manz M, Jung S, Sieweke M, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science*. 2010; 327:656–661. [PubMed: 20133564]

2. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol.* 2007; 7:292–304. [PubMed: 17380158]
3. Hume DA. Differentiation and heterogeneity in the mononuclear phagocyte system. *Mucosal Immunol.* 2008; 1:432–441. [PubMed: 19079210]
4. Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature.* 2003; 423:356–361. [PubMed: 12748655]
5. Kinne RW, Stuhlmuller B, Burmester GR. Cells of the synovium in rheumatoid arthritis: macrophages. *Arthritis Res Ther.* 2007; 9:224–239. [PubMed: 18177511]
6. Herman S, Muller RB, Kronke G, Zwerina J, Redlich K, Hueber AJ, et al. Induction of osteoclast-associated receptor, a key osteoclast costimulation molecule, in rheumatoid arthritis. *Arthritis Rheum.* 2008; 58:3041–3050. [PubMed: 18821671]
7. Schett G. Cells of the synovium in rheumatoid arthritis: osteoclasts. *Arthritis Res Ther.* 2007; 9:203. [PubMed: 17316459]
8. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* 2008; 8:958–969. [PubMed: 19029990]
9. Zvaifler NJ. The immunopathology of joint inflammation in rheumatoid arthritis. *Adv Immunol.* 1973; 16:265–336. [PubMed: 4599390]
10. Van Lent PL, Grevers L, Lubberts E, de Vries TJ, Nabbe KC, Verbeek S, et al. Fc γ receptors directly mediate cartilage, but not bone, destruction in murine antigen-induced arthritis: uncoupling of cartilage damage from bone erosion and joint inflammation. *Arthritis Rheum.* 2006; 54:3868–3877. [PubMed: 17133594]
11. Sutterwala FS, Noel GJ, Salgame P, Mosser DM. Reversal of proinflammatory responses by ligating the macrophage Fc γ receptor type I. *J Exp Med.* 1998; 188:217–222. [PubMed: 9653099]
12. Bazin R, Lemieux R, Tremblay T, St-Amour I. Tetramolecular immune complexes are more efficient than IVIg to prevent antibody-dependent in vitro and in vivo phagocytosis of blood cells. *Br J Haematol.* 2004; 127:90–96. [PubMed: 15384982]
13. Park-Min KH, Serbina NV, Yang W, Ma X, Krystal G, Neel BG, et al. Fc γ RIII-dependent inhibition of interferon- γ responses mediates suppressive effects of intravenous immune globulin. *Immunity.* 2007; 26:67–78. [PubMed: 17239631]
14. Siragam V, Crow AR, Brinc D, Song S, Freedman J, Lazarus AH. Intravenous immunoglobulin ameliorates ITP via activating Fc γ receptors on dendritic cells. *Nat Med.* 2006; 12:688–692. [PubMed: 16715090]
15. Siragam V, Brinc D, Crow AR, Song S, Freedman J, Lazarus AH. Can antibodies with specificity for soluble antigens mimic the therapeutic effects of intravenous IgG in the treatment of autoimmune disease? *J Clin Invest.* 2005; 115:155–160. [PubMed: 15630455]
16. Silverman GJ, Goodyear CS, Siegel DL. On the mechanism of staphylococcal protein A immunomodulation. *Transfusion.* 2005; 45:274–280. [PubMed: 15660839]
17. Hanson DC, Schumaker VN. A model for the formation and interconversion of protein A-immunoglobulin G soluble complexes. *J Immunol.* 1984; 132:1397–1409. [PubMed: 6693770]
18. Langone J, Das C, Mainwaring R, Shearer W. Complexes prepared from protein A and human serum, IgG, or Fc γ fragments: characterization by immunochemical analysis of ultracentrifugation fractions and studies on their interconversion. *Mol Cell Biochem.* 1985; 65:159–170. [PubMed: 3157047]
19. Das C, Langone J. Correlation between antitumor activity of protein A and in vivo formation of defined high molecular weight complexes with immunoglobulin G in BALB/c mice. *Cancer Res.* 1987; 47:2002–2007. [PubMed: 3828988]
20. Goodyear CS, Silverman GJ. Staphylococcal toxin induced preferential and prolonged in vivo deletion of innate-like B lymphocytes. *Proc Natl Acad Sci U S A.* 2004; 101:11392–11397. [PubMed: 15273292]
21. Edwards JP, Zhang X, Frauwirth KA, Mosser DM. Biochemical and functional characterization of three activated macrophage populations. *J Leukoc Biol.* 2006; 80:1298–1307. [PubMed: 16905575]

22. Leung BP, McInnes IB, Esfandiari E, Wei XQ, Liew FY. Combined effects of IL-12 and IL-18 on the induction of collagen-induced arthritis. *J Immunol.* 2000; 164:6495–6502. [PubMed: 10843707]
23. Simelyte E, Rosengren S, Boyle DL, Corr M, Green DR, Firestein GS. Regulation of arthritis by p53: critical role of adaptive immunity. *Arthritis Rheum.* 2005; 52:1876–1884. [PubMed: 15934085]
24. Joosten LA, Lubberts E, Durez P, Helsen MM, Jacobs MJ, Goldman M, et al. Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis: protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction. *Arthritis Rheum.* 1997; 40:249–260. [PubMed: 9041936]
25. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008; 3:1101–1108. [PubMed: 18546601]
26. Willcocks LC, Smith KG, Clatworthy MR. Low-affinity Fc γ receptors, autoimmunity and infection. *Exp Rev Mol Med.* 2009; 11:e24.
27. Tierney JB, Kharkrang M, La Flamme AC. Type II-activated macrophages suppress the development of experimental autoimmune encephalomyelitis. *Immunol Cell Biol.* 2009; 87:235–240. [PubMed: 19104504]
28. Silverman GJ, Goodyear CS. Confounding B-cell defences: lessons from a staphylococcal superantigen. *Nat Rev Immunol.* 2006; 6:465–475. [PubMed: 16724100]
29. Yanaba K, Hamaguchi Y, Venturi GM, Steeber DA, St Clair EW, Tedder TF. B cell depletion delays collagen-induced arthritis in mice: arthritis induction requires synergy between humoral and cell-mediated immunity. *J Immunol.* 2007; 179:1369–1380. [PubMed: 17617630]
30. Anderson CF, Mosser DM. Cutting edge: biasing immune responses by directing antigen to macrophage Fc γ receptors. *J Immunol.* 2002; 168:3697–3701. [PubMed: 11937518]
31. Boissier MC, Chiochia G, Bessis N, Hajnal J, Garotta G, Nicoletti F, et al. Biphasic effect of interferon- γ in murine collagen-induced arthritis. *Eur J Immunol.* 1995; 25:1184–1190. [PubMed: 7774621]
32. Massey HM, Flanagan AM. Human osteoclasts derive from CD14-positive monocytes. *Br J Haematol.* 1999; 106:167–170. [PubMed: 10444181]
33. Lader CS, Scopes J, Horton MA, Flanagan AM. Generation of human osteoclasts in stromal cell-free and stromal cell-rich cultures: differences in osteoclast CD11c/CD18 integrin expression. *Br J Haematol.* 2001; 112:430–437. [PubMed: 11167844]
34. Sulica A, Medesan C, Laky M, Onica D, Sjoquist J, Ghetie V. Effect of protein A of *Staphylococcus aureus* on the binding of monomeric and polymeric IgG to Fc receptor-bearing cells. *Immunology.* 1979; 38:173–179. [PubMed: 511216]
35. Silverman GJ, Nayak JV, Warnatz K, Hajjar FF, Cary S, Tighe H, et al. The dual phases of the response to neonatal exposure to VH family-restricted staphylococcal B cell superantigen. *J Immunol.* 1998; 161:5720–5732. [PubMed: 9820554]
36. Goodyear CS, Sugiyama F, Silverman GJ. Temporal and dose-dependent relationships between in vivo B cell receptor-targeted proliferation and deletion-induced by a microbial B cell toxin. *J Immunol.* 2006; 176:2262–2271. [PubMed: 16455982]
37. Goodyear CS, Silverman GJ. Death by a B cell superantigen: in vivo VH-targeted apoptotic supraclonal B cell deletion by a Staphylococcal toxin. *J Exp Med.* 2003; 197:1125–1139. [PubMed: 12719481]
38. Goodyear CS, Corr M, Sugiyama F, Boyle DL, Silverman GJ. Cutting edge: Bim is required for superantigen-mediated B cell death. *J Immunol.* 2007; 178:2636–2640. [PubMed: 17312102]
39. Poullin P, Announ N, Mugnier B, Guis S, Roudier J, Lefevre P. Protein A-immunoabsorption (Prosorba column) in the treatment of rheumatoid arthritis. *Joint Bone Spine.* 2005; 72:101–103. [PubMed: 15797486]
40. Brunner J, Kern P, Gaip U, Munoz L, Voll R, Kalden J, et al. The low-throughput protein A adsorber: an immune modulatory device. Hypothesis for the mechanism of action in the treatment of rheumatoid arthritis. *Mod Rheumatol.* 2005; 15:9–18. [PubMed: 17028816]

41. Sasso EH, Merrill C, Furst DT. Is release of staphylococcal protein A (SPA) during immunoadsorption therapy of rheumatoid arthritis related to clinical response? *Arthritis Rheum.* 2000; 43(Suppl):S290. [abstract].
42. Hanson DC, Phillips ML, Schumaker VN. Electron microscopic and hydrodynamic studies of protein A-immunoglobulin G soluble complexes. *J Immunol.* 1984; 132:1386–1396. [PubMed: 6693769]
43. Gerber JS, Mosser DM. Reversing lipopolysaccharide toxicity by ligating the macrophage Fc γ receptors. *J Immunol.* 2001; 166:6861–6868. [PubMed: 11359846]
44. Bernstein KE, Alexander CB, Mage RG. Nucleotide sequence of a rabbit IgG heavy chain from the recombinant F–I haplotype. *Immunogenetics.* 1983; 18:387–397. [PubMed: 6313520]
45. Johansson PJ, Myhre EB, Blomberg J. Specificity of Fc receptors induced by herpes simplex virus type 1: comparison of immunoglobulin G from different animal species. *J Virol.* 1985; 56:489–494. [PubMed: 2997471]
46. Antonsson A, Johansson P. Binding of human and animal immunoglobulins to the IgG Fc receptor induced by human cytomegalovirus. *J Gen Virol.* 2001; 82:1137–1145. [PubMed: 11297688]
47. Ravetch, JV.; Nimmerjahn, F. Fc receptors. In: Paul, WE., editor. *Fundamental immunology.* 5th ed.. Philadelphia: Lippincott-Raven; 2008. p. 684-705.
48. Gravalles EM, Harada Y, Wang JT, Gorn AH, Thornhill TS, Goldring SR. Identification of cell types responsible for bone resorption in rheumatoid arthritis and juvenile rheumatoid arthritis. *Am J Pathol.* 1998; 152:943–951. [PubMed: 9546355]
49. Torbinejad M, Clagett J, Engel D. A cat model for the evaluation of mechanisms of bone resorption: induction of bone loss by simulated immune complexes and inhibition by indomethacin. *Calcif Tissue Int.* 1979; 29:207–214. [PubMed: 117884]

Figure 1.

Staphylococcal protein A (SPA) binds to circulating monocytes and tissue macrophages in mice with collagen-induced arthritis. Mice were injected with 500 μ g of Alexa Fluor 488–labeled SPA (SPA-488) or ovalbumin (OVA-488), and 2 hours later, the blood and spleens were harvested for cell analyses. **A**, Representative results of flow cytometry are shown. Monocytes and neutrophils from the blood were initially gated based on their forward light-scatter (FSc) versus side light-scatter (SSc) patterns (top left panel), and then gated by lymphocyte antigen (Ly-6C versus Ly-6G/C) expression (top right panels). The lower panels show binding of OVA-488 or SPA-488 to CD11b⁺ monocytes and neutrophils. Values in

the boxed areas (same as boxed areas in the top panels) are the percentage of positive cells in each population. **B** and **C**, The extent of OVA-488 or SPA-488 binding was determined in monocytes and neutrophils (**B**) and in splenic CD11b+ macrophages (**C**). Bars show the mean $\pm$ SD representative results from 1 of 3 independent experiments, expressed as the mean fluorescence intensity (MFI) in 3 mice per group. *** = $P < 0.001$ versus OVA-488, by t -test. NS = not significant. **D**, Mice of the μ MT strain were injected intravenously with phosphate buffered saline (PBS) or mouse polyclonal IgG. Mice were then injected intraperitoneally with 300 μ g SPA-488 or OVA-488, and 2 hours later, the blood was collected to determine the extent of binding of OVA-488 or SpA-488 to monocytes. Bars show the mean $\pm$ SD representative results from 1 of 2 independent experiments, expressed as the MFI in 2 mice per group.

Figure 2.

Formation of staphylococcal protein A (SPA)–IgG immune complexes (SICs) and the presence of Fc γ receptor type I (Fc γ RI) are required for binding to macrophages and preosteoclasts. Bone marrow cells from C57BL/6 and Fc γ RI^{-/-} mice were enriched for monocytes and cultured with recombinant macrophage colony-stimulating factor (M-CSF) or M-CSF plus RANKL for 5 days to generate bone marrow–derived macrophages (BMMs) or preosteoclasts, respectively. These cells were incubated with Alexa Fluor 488–labeled ICs (ovalbumin plus polyclonal IgG [OpIg-488], SPA-488, SIC-488, or ovalbumin-488 [not shown]) to evaluate binding. Representative images show the binding of each complex to CD11b⁺ BMMs and preosteoclasts. Values over the boxed populations are the percentage of positive cells. The data shown are representative flow cytometry results, tested in duplicate, from 1 of 3 independent experiments.

Figure 3.

Treatment of mice with staphylococcal protein A (SPA)-IgG immune complexes (SICs) alters the cytokine profile and activation state of macrophages in an Fc γ receptor type I (Fc γ RI)-dependent manner. **A**, Bone marrow-derived macrophages (BMMs) from DBA1/J mice were stimulated for 6 hours without or with lipopolysaccharide (LPS) in the absence or presence of SICs, and levels of interleukin-10 (IL-10) and IL-12p40 were determined. Stimulation with IgG did not yield results significantly different from those with either medium alone or SPA, and therefore IgG is not shown. **B** and **C**, BMMs from C57BL/6 mice were primed with interferon- γ , and 16 hours later, the cells were stimulated with LPS (Ca-M Φ), SICs + LPS (SIC-M Φ), or ovalbumin ICs (M Φ -II) (21) or left unstimulated (Uns-M Φ). Six hours later, the BMMs were analyzed by flow cytometry for the expression of class II major histocompatibility complex (MHC II) and CD86, as shown in representative histograms (**B**) and shown as the mean fluorescence intensity (MFI) (**C**). Incubations over 24 hours (not shown) resulted in the same expression profiles. **D**, BMMs from C57BL/6 and Fc γ RI^{-/-} mice were stimulated for 6 hours with LPS in the absence or presence of SICs. Results with IgG + LPS and SPA + LPS did not differ significantly from those with LPS alone, and therefore these are not shown. Bars show the mean $\pm$ SD representative results from 1 of 3 independent experiments, determined in samples from triplicate wells, measured in triplicate. * = $P < 0.05$; *** = $P < 0.001$, by analysis of variance with post hoc test.

Figure 4.

Treatment of mice with staphylococcal protein A (SPA) at disease onset inhibits the development of arthritis. Mice with collagen-induced arthritis were treated on day 25 after induction of arthritis and every other day thereafter with either vehicle or SPA. **A** and **B**, The severity (**A**) and incidence (**B**) of arthritis were assessed in each group. Bars in **A** and **B** show the mean $\pm$ SEM results (pooled from 2 experiments) in 16–17 mice per group. * = $P = 0.02$ versus vehicle, by two-way repeated-measures analysis of variance (ANOVA) in **A** or by log-rank test in **B**. **C** and **D**, Representative histologic images of hematoxylin and eosin (H&E)–stained knees from vehicle-treated (**C**) and SPA-treated (**D**) mice on day 42 after arthritis induction are shown. **E**, Histologic features of arthritis (assessed as hyperplasia, infiltration, and erosion in H&E-stained knees, and cartilage destruction, as measured by proteoglycan [PG] depletion, in Safranin O–stained sections) were scored in a blinded manner. Bars show the mean $\pm$ SD of 9 mice per group. ** = $P < 0.01$; *** = $P < 0.001$ versus vehicle, by ANOVA with post hoc test. **F** and **G**, Knees from vehicle-treated (**F**) and SPA-treated (**G**) mice were stained for cathepsin K–positive cells (osteoclasts) (indicated by the red arrow) on day 42 after arthritis induction. Representative images are shown. **H**, Sections stained for cathepsin K were measured for bone area and the number of osteoclasts (cathepsin K–positive cells) per mm^2 of bone was determined. Bars show the mean $\pm$ SD of 5–8 mice per group. *** = $P < 0.001$ versus vehicle, by t -test.

Figure 5.

Treatment of mice with staphylococcal protein A (SPA) enhances the proliferative responses to CB11 peptide, increases collagen-specific IgG1 antibodies, and renders macrophages unresponsive to interferon- γ (IFN γ). Mice with established collagen-induced arthritis (CIA) were treated on day 25 with intraperitoneal (IP) injections of either vehicle alone or 0.1 mg of SPA every other day. **A**, Inguinal lymph nodes collected on day 42 were cultured in medium alone or with 30 $\mu\text{g}/\text{ml}$ CB11 peptide. The recall response to CB11 peptide was calculated as the counts per minute of ^3H -thymidine (^3H -TdR) incorporation in medium alone subtracted from the peptide-specific counts. Bars show the mean $\pm$ SEM of 7 mice per

group. **B–D**, Serum levels of anticollagen (anti-CII) mouse IgG2a (**B**) and IgG1 (**C**), expressed as relative units (RU), and serum levels of interleukin-10 (IL-10) and IL-12 (**D**) were determined on the day of harvest (day 42). There were no significant differences in the total levels of IgG (data not shown). In **B** and **C**, results are shown as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles, for 9–10 mice per group. In **D**, results are the mean $\pm$ SD of 5 mice per group. **E**, Mice with established CIA in each treatment group were treated on day 42 with an IP injection of IFN γ or vehicle (v). Peritoneal macrophages were harvested 3 hours later, RNA was extracted, and quantitative polymerase chain reaction was performed to determine the expression of IP10 and IRF1. Values for the target genes were normalized to those for GAPDH, with results expressed as the mean $\pm$ SD fold change in gene expression compared to vehicle in 7–9 mice per group. * = $P < 0.05$ versus vehicle, by t -test; ** = $P < 0.01$ and *** = $P < 0.001$ versus vehicle, by analysis of variance with post hoc test.

Figure 6.

Staphylococcal protein A (SPA)-IgG immune complexes (SICs) alter the activation state of human macrophages and inhibit human preosteoclast differentiation into osteoclasts. **A**, Human CD14⁺ monocytes were cultured with macrophage colony-stimulating factor (M-CSF) without or with SICs, and on day 7, the cells were stimulated with lipopolysaccharide (LPS). Levels of tumor necrosis factor α (TNF α) and interleukin-12 (IL-12) were determined at 6 hours and 24 hours, respectively. The level of secretion with LPS alone (medium) was defined as 100%. The results in cultures with SPA were not significantly different from those with IgG or medium alone, and therefore SPA is not shown. Bars show the mean $\pm$ SD results from triplicate wells ($n = 4$ per group). * = $P < 0.05$; *** = $P < 0.001$ versus IgG, by analysis of variance with post hoc test. **B**, Differentiated human macrophages were treated on day 6 with either medium, SPA, IgG, or SICs at 25 μ g/ml for 48 hours. Cultures were left unstimulated or stimulated with 10 units/ml of interferon- γ (IFN γ) 10 minutes prior to lysis. Relative values of phosphorylated STAT-1 (pY-STAT-1) were normalized to those for the total STAT-1 signal by densitometric quantitation (non-IFN γ treated = 1); normalized values are shown below the blots. β -actin was used as a control. **C** and **D**, Human macrophages were incubated with either medium (**C**) or SICs (**D**) for 48 hours, followed by 10 units/ml of IFN γ 10 minutes prior to fixation, and the cells were stained for phosphorylated STAT-1 (pY-STAT-1) (in red) and nuclei (in blue). No pY-STAT-1 staining was observable in cells that did not receive IFN γ . Images show representative results from 1 of 3 separate experiments. **E**, Human CD14⁺ monocytes ($n = 6$) were cultured with RANKL and M-CSF overnight and treated with SICs for 6 hours. Expression of CD115 was assessed as the mean fluorescence intensity (MFI). ** = $P < 0.01$ by paired t -test. **F**, Human CD14⁺ monocytes ($n = 7$) were cultured with RANKL and M-

CSF in the presence of medium, IgG, ovalbumin plus polyclonal IgG (OpIg), SPA, or SICs starting on day 1. On day 7, cells were counted to determine the number of tartrate-resistant acid phosphatase–positive multinucleated cells (TRAP+ MNC) (defined as ≥ 3 nuclei). Bars show the mean $\pm$ SD. ** = $P < 0.01$ versus vehicle, by Mann-Whitney U test. **G**, Representative images of osteoclast cultures on day 7 after treatment with medium, IgG, OpIg, or SIC are shown.