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Intra-peritoneal sRAGE treatment induces alterations in cellular distribution of $CD19^+$, $CD3^+$ and $Mac\text{-}1^+$ cells in lymphoid organs and peritoneal cavity

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ABBREVIATIONS:
AGE - Advanced glycation end products
BAFF - B cell activating factor
DAMP - Damage-associated molecular pattern
HMGB1 - High mobility group chromosomal protein 1
IF - Interferon
IL - Interleukin
LPS - Lipopolysaccharide
RAGE - Receptor for advanced glycation end products

ABSTRACT

Receptor for advanced glycation end products (RAGE) is a pattern recognition receptor that binds a variety of pro-inflammatory ligands. Its soluble form, sRAGE, can compete for ligand binding and thereby have an anti-inflammatory effect. We have recently reported that sRAGE also exerts pro-inflammatory and chemotactic properties suggesting a dual role for sRAGE in immune modulation. Our present aim was to analyse the immunomodulatory properties of sRAGE in vivo with respect to acquired immunity. Naïve mice were treated intra-peritoneally with sRAGE and cells from peritoneal lavage, spleens and bone marrow were examined. Mice treated with sRAGE displayed an increased leucocyte count in the peritoneal cavity, enlarged spleens and increased cellularity compared with vehicle-treated. Furthermore, sRAGE-treatedmice had a significantly increased frequency and number of CD19+ B cells in spleen and a reduced frequency of CD19+ B cells in bone marrow compared with controls. Functionally, splenocytes from sRAGE-treated mice showed elevated IgG production and up to a four-fold increased IgM secretion compared with control animals and produced significantly higher levels of interleukin-10, interferon-γ and interleukin-6 in response to lipopolysaccharide stimulation. Our results suggest that sRAGE has immunomodulatory properties, since intra-peritoneal administration of sRAGE into healthy mice leads to rearrangements in cellular composition in the bone marrow and spleen. Moreover, the administration of sRAGE directs B cells into the spleen and towards differentiation. Our novel findings indicate that sRAGE exerts an effect on the cells of adaptive immunity.

INTRODUCTION

The full-length receptor for advanced glycation end products (RAGE) is a multi-ligand member of the immunoglobulin superfamily, being expressed as a cell surface molecule and playing a key role in diverse inflammatory processes (Schmidt et al. 2000). The receptor protein is composed of three immunoglobulin-like regions, a transmembrane domain and a highly charged short cytosolic tail that is essential for intracellular signalling. The V-domain in the extra-cellular part of the receptor protein is critical for ligand-binding and interacts with a diverse class of ligands because of its ability to recognize three-dimensional structures rather than specific aminoacid sequences (Schmidt et al. 2001). Many non-glycated endogenous ligands of RAGE, including damage-associated molecular patterns (DAMPs), have been identified in addition to initially recognized advanced glycoxidation end products (AGEs; Foell et al. 2007a, 2007b). RAGE is thus considered a pattern recognition receptor and has been shown to interact with an endogenous alarmin high mobility group box chromosomal protein 1 (HMGB1), certain members of the S100/calgranulin family of pro-inflammatory cytokines, the amyloid-β peptide and β-fibrils and the β2-integrin Mac-1 (Chavakis et al. 2003; Hofmann et al. 1999; Lotze and Tracey2005). The common denominator for all these ligands is generation and their over expression in diverse pathophysiological and inflammatory conditions (Bucciarelli et al. 2002). Engagement of RAGE by these ligands leads ultimately to the amplification of the inflammatory response via a selfamplifying positive-feedback loop (Bucciarelli et al. 2002).

RAGE is expressed at low levels in normal tissues and vasculature, except for in the lung in which it is abundant (Brett et al. 1993). However, during inflammatory and pathophysiological conditions, whereas RAGE becomes upregulated, cells can release diverse soluble isoforms of RAGE (sRAGE) into the extracellular space (Kalea et al. 2012; Vazzana et al. 2009). Soluble RAGE either can arise as a product from alternative mRNA splicing or is

produced by the cleavage of the extracellular region of the membrane-associated receptor by sheddases belonging to ADAM (a disintegrin and metalloproteinases) family. Most of the circulating sRAGE (ca. 80%) in humans derives from the ectodomain shedding of cell-surface RAGE (Yamagishi and Matsui 2010), and its level might correlate with ongoing inflammation (Raucci et al. 2008).

Soluble RAGE, spanning the ligand-binding domain, competes with cell-bound RAGE for ligand binding and functions as a "decoy" abrogating cellular activation and therefore protecting against chronic inflammation. As evidence, recombinant sRAGE has been successfully used in several animal models and shown to reduce inflammatory responses in collagen-type-II-induced arthritis (Hofmann et al. 2002), delayed type hypersensitivity (Hofmann et al. 1999; Liliensiek et al. 2004), experimental autoimmune encephalomyelitis (Yan et al. 2003) and diabetic atherosclerosis (Goova et al. 2001; Park et al. 1998). In all these models of chronic inflammation, a wide diversity of pro-inflammatory RAGE ligands is present and, by competing with membrane-bound RAGE for ligand binding, sRAGE blocks cellular activation. The net effect of sRAGE administration in these cases is anti-inflammatory.

However, the endogenous activity and properties of sRAGE are not fully understood. Recent studies have raised the question as to whether sRAGE also has an immune-modulating activity of its own. We have recently shown that sRAGE treatment down-regulates HMGB1-induced arthritis (Pullerits et al. 2006). Indeed, the incidence and the severity of arthritis induced by intra-articular injection of HMGB1, a potent pro-inflammatory cytokine, decreases significantly in mice receiving intraperitoneal sRAGE treatment (Pullerits et al. 2006). Surprisingly, peritoneal leucocyte counts have revealed a several-fold increased extravasation of inflammatory cells into the peritoneum of sRAGE-treated mice. We have further observed that sRAGE possesses pro-inflammatory and chemotactic properties that are mediated through interaction with β2 integrin Mac-1 and, further downstream, by nuclear factor kappa B (NF-κB) activation (Pullerits et al. 2006). Our findings have recently been confirmed by Wang et al. (2010). They have reported that sRAGE induces monocyte and neutrophil migration in vitro and have suggested a novel role for sRAGE in monocyte-neutrophil-mediated inflammation and mononuclear phagocyte survival and differentiation (Wang et al. 2010). In addition, exposure to sRAGE has also been shown to decrease the delayed-type hypersensitivity response in RAGE knockout mice indicating that sRAGE has properties other than purely blocking cell surface RAGE function (Liliensiek et al. 2004).

We have undertaken this study to further analyse the immunomodulatory properties of sRAGE in vivo and in vitro with respect to acquired immunity by using a healthy mouse model.

MATERIALS AND METHODS

Mice

Female Naval Medical Research Institute (NMRI) mice, 6–8 weeks old, were housed in the animal facility of the Department of Rheumatology and Inflammation Research, University of Gothenburg. They were kept under standard conditions of temperature and light and fed laboratory chow and water ad libitum. The study was approved by the Ethical Committee of Gothenburg University and the regulations of the National Board for Laboratory Animals were followed.

Injection and treatment protocol

Mice were treated with the established dose of 100 μg sRAGE daily/mouse intra-peritoneally or the same volume of phosphate-buffered saline (PBS) as previously described (Pullerits et al. 2006). Following 4 days of treatment, the mice were killed and blood, bone marrow, spleen and peritoneal lavage samples were obtained. The experiment was repeated twice with 4–5 mice in each group.

Reagents

Lipopolysaccharide (LPS) from Escherichia coli serotype 055:B5 and concanavalin A (ConA) were purchased from Sigma (St. Louis, Mo., USA). Endotoxin-free sRAGE was kindly provided by Professor A.-M. Schmidt (NYU Langone Medical Center) and prepared as previously described (Park et al. 1998). Poke weed mitogen (PWM) was purchased from E.Y. Laboratories (San Mateo, Calif., USA).

Haematological analyses

To evaluate leucocyte recruitment into the peritoneum, peritoneal lavage was performed with 2 ml ice cold PBS followed by re-aspiration. Samples of peritoneal lavage were thereafter centrifuged at 1,200 rpm for 5 min; cell pellets were dissolved with an equal amount of PBS and total leucocyte counts were determined by using a cell counter (Sysmex KX-21, Toa Medical Electronics, Kobe, Japan).
For bone marrow sampling, femurs were carefully dissected. A hole was made at each end of the bone and 2 ml cold PBS was forced through. Samples were thereafter centrifuged at 1,200 rpm for 5 min at 4°C and resuspended in NH4Cl solution (0.83%, pH 7.29) on ice for 7 min to lyse erythrocytes, followed by two washing steps in cold PBS. Cell counts were determined in a cell counter. Spleens isolated from mice were pushed through a 70-μm cell strainer and red blood cells were lysed as described (Amu et al. 2006). Cells were washed in PBS and resuspended for cell counting.

In vitro experiments

Spleens from NMRI mice treated with sRAGE or control vehicle were weighed under sterile conditions. The cellular cultures were prepared as previously described (Pullerits et al. 2005) and stimulated with final concentrations of 2.5 μg/ml ConA (T cell mitogen), 1.0 μg/ml PWM (T-cell-dependent B cell mitogen) or 1 μg/mg LPS (B cell mitogen). Cell culture supernatants were collected after 24 and 48 h for determination of cytokine levels. For in vitro proliferation assay, cells were incubated for 72 h in 96-well plates with final concentrations of 2.5 μg/ml ConA, 1.0 μg/ml PWM or 1.0 μg/ml LPS. Culture medium was used as a negative control. The cultures were pulsed with 1 μCi [3 H]-thymidine (Amersham Pharmacia Biotech) at 12 h before being harvested. The cells were harvested onto glass fibre filters (Walluc Oy, Turku, Finland) and dried. Thereafter, incorporated [3 H]-thymidine was measured by using a β-scintillation counter. The proliferative response was expressed as mean±SEM (median) counts per minute in triplicate samples from five spleens in each group.

ELISPOT assay for evaluation of immunoglobulin production

Plates with 96 wells (Millipore, Billerca, Mass., USA) were coated with affinity-purified goat F(ab')2 fragments specific for mouse Ig (H + L) (MP Biomedicals, Aurora, Ohio, USA) at

0.25 μg per well overnight at 4°C. After being washed with PBS, plates were blocked with 5% fetal calf serum (FCS) in PBS for 1 h at room temperature. Samples (50 μl) of isolated cells from spleen and bone marrow were added in triplicate at a concentration of 106 and 105 cells/ml to Iscove's medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 1% gentamycin (Sigma-Aldrich) and 1%L-glutamine (Sigma-Aldrich) followed by incubation in a humidified atmosphere containing 5% CO2 at 37°C for 4 h. After the cells had been washed, alkaline-phosphatase-labeled goat anti-mouse IgG and IgM (Southern Biotechnology, Birmingham, Ala., USA) were added at optimal concentration and the plates were incubated overnight at 4°C. After another washing step, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Bio-Rad Laboratories, Hercules, California, USA) was added for 20–30 min at room temperature. Spots were counted by using a microscope and the results were presented as spot-forming cells (SFC) per 106 cells.

Enzyme-linked immunosorbent assay for detection of total immunoglobulin levels

Enzyme-linked immunosorbent assay (ELISA) plates (Nunc, Roskilde, Denmark) were coated with 2 μg/ml goat affinitypurified F(ab')2 fragments to mouse IgG and IgM (MP Biomedicals, Santa Ana, Calif., USA). Samples were diluted by serial dilution and detected with 0.5 μg/ml biotin-SP conjugated affinity-purified F(ab')2 fragments of goat-antimouse IgG or IgM (Jackson Immunoresearch, Suffolk, UK). The ELISA was developed by using 0.5 μg/ml extravidin-peroxidase (Sigma Aldrich) followed by TMB substrate (Sigma Aldrich).

Flow cytometry analysis

For flow cytometry (FACS) analysis, 105–106 cells were placed in 96-well plates and pelleted (3 min, 300g, 4°C). To avoid unspecific binding via Fc-receptor interactions, cells were incubated with Fc block (2,4 G2, BD Bioscience) as previously described (Amu et al. 2006). Samples labeled with goat anti-mouse IgG and its matched control were incubated with 0.1% goat serum instead of Fc block for 10 min at room temperature. Following the blocking of the Fc-receptors, cells were stained as previously described (Amu et al. 2006) and thereafter 2×104 to 1×105 cells were collected from each sample by using a FACSCantoII (BD Bioscience) with FACSDiva software. The instrument was set by using unstained and single stained spleen cells, and data analyses were performed by using FlowJo software, (Tree Star, Ashland, Ore., USA). All data in the study are presented as levels above background. All antibodies were diluted in FACS-buffer to optimal concentrations after titration experiments. The antibodies used were either directly conjugated with fluorescein isothiocyanate (FITC), r-phycoerythin (PE), peridinin chlorophyllprotein complex (PerCp) or biotin. Antibodies conjugated with biotin were further exposed to streptavidin conjugated either with PE or allophycocyanin (APC). The employed antibodies included anti-CD3e (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD19 (1D3), anti-mouse MAC1 (M1/70), anti-mouse IgA (C10-1), anti-mouse IgD (11-26c.2a) or anti-mouse IgM (R6-60.2), all of which were purchased from BD Bioscience, and anti-mouse IgG purchased from Jackson Immuno Research Laboratories. The specific isotype controls for all antibodies were run at the same time. Isotype controls used were hamster IgG1, κ (A19-3), hamster IgG1/λ (A111-3), rat IgG1, λ (A110-1), rat IgG1, κ (R3-34), rat IgG2a, κ (R35-95), rat IgG2b, κ (A95-1), or rat IgM, κ (R4-22), which were all from BD Bioscience, and normal goat IgG from RD systems.

Determination of cytokine and chemokine levels

The levels of interleukin-10 (IL-10), IL-6, IL-4 and interferon-γ (IFN-γ) were measured in mice sera and cell culture supernatants by using the Cytometric Bead Array (CBA) Mouse Soluble Protein Flex Set system (BD
Bioscience) with the capture beads was employed according to the manufacturer's recommendations and as previously described (Amu et al. 2010). The levels of B cell activating factor (BAFF) were determined by using a specific sandwich ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's protocol.

Statistical analysis

Non-parametric methods were used for statistical comparisons, since data were not normally distributed. Statistical differences between independent groups were calculated by using the Kruskal-Wallis test followed by the Mann–Whitney U-test and Fisher's exact probability test, as appropriate. All values are reported as median (25th–75th percentiles). Statistical analyses were performed by using StatView version 5.0.1.0 (SAS Institute). A P-value below 0.05 was considered significant.

RESULTS

Soluble RAGE causes influx of leucocytes into peritoneal cavity and leads to splenomegaly

We recently reported that intra-peritoneal administration of sRAGE down-regulated HMGB1-induced arthritis via a deviation of the inflammatory response from HMGB1-injected joints to the peritoneal cavity (Pullerits et al. 2006). Based on our previous findings, we wanted to investigate further the immunomodulatory properties of sRAGE in vivo.

Naive NMRI mice were treated systemically with 100 μg sRAGE once a day via an intra-peritoneal route for four consecutive days as previously described (Pullerits et al.2006). The control group received similar treatment with a corresponding volume of PBS. Following treatment, mice were killed and spleen, bone marrow, peritoneal lavage and blood samples were collected.

In accordance with our previous study (Pullerits et al. 2006) the assessment of leucocyte counts in the peritoneal lavage fluids revealed that sRAGE administration induced a three-fold increase of cells in the peritoneal cavity (median 10.4 [7.0–11.1]×10^6 versus 3.6 [2.7–4.2]× 10^6, P=0.009) in comparison with vehicle injections. We observed a significantly increased frequency of CD3+ cells in the peritoneal cavity of sRAGE-treated mice (median 13.7 [11.4%–16.2%] versus 4.7 [4.3%–6.9%], P=0.028), whereas the frequency of Mac-1+ cells was lower compared with PBS-treated animals (median 41.3 [36.5%–44.4%] versus 48.9 [47.4%–58.9%], respectively, P=0.047). No significant difference was seen regarding CD19+ cells between the groups (Fig.1a). The absolute number of all cell types including CD3+, CD19+ and Mac-1+ cells was significantly elevated in sRAGE-treated mice (Fig. 1b). No difference was seen between the groups with regard to the total white blood cell count in circulation and bone marrow (data not shown).

Interestingly, we found that systemic treatment with sRAGE led to splenomegaly. Mice receiving sRAGE displayed significantly enlarged spleens (median 170 [167– 186] mg versus 126 [120–150] mg, P=0.0009) and also an increased cellularity (total

number of splenocytes: 104.8 [92.0–125.4]×10^6 versus 70.4 [58.4–100.1]× 10^6, P=0.015) compared with PBS-treated animals, respectively (Fig.1c, d).

Soluble RAGE treatment changes cellular composition in spleen and bone marrow

We further investigated the cell type that was involved in the sRAGE-induced splenomegaly by using FACS analysis. Cells from spleen and bone marrow were collected and stained for antigens specific for B cells (CD19), T cells (CD4 and CD8) and macrophages (Mac-1).

In mice receiving intra-peritoneal injections of sRAGE, 52.1% [49.4%–53.1%] of splenocytes were CD19+ B cells in comparison with 44.4% [43.2%–48.5%] in control animals (P=0.012; Fig. 2a). sRAGE treated mice also displayed significantly higher total number of B cells (59.9 [47.2–65.0]× 10^6versus 33.5 [23.0–40.6]×10^6, respectively, P=0.002) in the spleen (Fig. 2b).We additionally observed a tendency towards an increase of Mac-1+ cells (7.3 [6.0–10.7]×10^6 versus 5.4 [3.1–6.9]×10^6, respectively, P=0.058) in sRAGE-treated compared with PBS-treated animals (Fig. 2b).

Analysis of bone marrow samples revealed that sRAGE treated animals had significantly higher frequencies ofMac-1+ cells (median 63.1 [62.7%–66.4%] versus 42.2 [41.8%–45.0%], P=0.009). In contrast, sRAGE treatment reduced the frequency of CD19+ B cells (median 13.3 [12.1%–16.9%] versus 24.0 [23.0%–28.5%], P=0.009) found in these animals (Fig. 2c). Furthermore, a significantly lower total number of bone marrow B cells was observed in mice receiving sRAGE versus controls (median 2.0 [1.9–2.7]×10^6 versus 4.8 [3.0– 5.0]×10^6, respectively, P=0.028; Fig. 2d). No significant changes were seen regarding frequency and total number of CD4+ and CD8+ cells in the spleen and bone marrow (Fig. 2a–d). Taken together, these results indicate that sRAGE treatment led to the re-arrangements in cellular composition in the lymphoid organs and peritoneal cavity.

Soluble RAGE modifies cytokine profile in splenocytes and mouse sera

Based on our previous findings that sRAGE dose-dependently induced production of pro-inflammatory cytokines in vitro (Pullerits et al. 2006), we further explored whether sRAGE treatment in vivo modulated the cytokine response to various mitogens. Mouse splenocytes from sRAGE-treated and control animals were stimulated with LPS, ConA or PWM. Levels of IL-10, IFN-γ, IL-4 and IL-6 were measured in the cell culture supernatants following a 24-h stimulation.

We observed that splenocytes from sRAGE-treated mice produced significantly higher amounts of IL-10, IL-6 and IFN-γ in response to stimulation with B cell mitogen LPS compared with control animals (Fig. 3a–c). In addition, increased spontaneous production of IFN-γ and IL-4 was seen in sRAGE-treated group compared with PBS-treated animals (Fig. 3a, d).

Additionally, an assessment of cytokines in the serum of mice treated with sRAGE revealed significantly higher levels of IFN-γ in their serum in comparison with the control group (median 1.5 [1.2–1.9 pg/ml] versus 0 [0–1.4 pg/ml], P=0.023, respectively). However, we could not detect any differences between the two groups regarding IL-10, IL-4 and IL-6 serum levels (data not shown).

To determine further whether sRAGE treatment induced monocytes/macrophages to secrete B cell-promoting cytokines such as BAFF, we also assessed BAFF levels in the sera of sRAGE-treated and control mice. Soluble RAGE-treated mice displayed similar serum levels of BAFF to those of vehicle treated controls (median 5986 [4434–7141] pg/ml versus 5823 [5718–6754] pg/ml, respectively; data not shown).

Soluble RAGE-treated mice display increased antibody production

Since sRAGE treatment caused enlargement of the spleen and changes in cellular composition in the bone marrow, we further wanted to investigate the functional properties of these cells.

We employed an ELISPOT assay to investigate the impact of sRAGE on immunoglobulin secretion. Spleen cells obtained from sRAGE-treated animals displayed significantly higher IgG production compared with splenocytes from control animals. Furthermore, the secretion of IgM was increased up to four-fold in comparison with PBS-treated animals (Fig. 4a). In contrast, no significant differences were detected between the groups regarding IgG and IgM secretion from cells isolated from bone marrow (data not shown).

However, an analysis of surface-bound immunoglobulin expression by FACS revealed that the frequency of IgG on CD19+ B cells in bone marrow was significantly higher (P=0.014) in sRAGE-treated animals than in control mice. In contrast, we did not observe any differences regarding surface-bound IgA, IgM and IgD expression on CD19+ B cells, either in spleen or in bone marrow. These observations indicate that administration of sRAGE induces differentiation of B cells, both in bone marrow and in spleen.

We also evaluated the effect of sRAGE treatment on immunoglobulin production in vivo. In corroboration with our in vitro results, mice treated intra-peritoneally with sRAGE had significantly higher total serum levels of IgM (Fig. 4b). No significant differences between groups were revealed regarding the production of total IgG levels (Fig. 4c).

Soluble RAGE treatment alters LPS-induced B cell proliferation

Spleen cells from sRAGE-treated and control mice were stimulated with various antigens to investigate the proliferative response. Interestingly, spleen cells obtained from sRAGE treated mice showed a significantly decreased proliferative response to B cell mitogen LPS compared with splenocytes from control animals (P=0.009; Fig. 5). T-cell-dependent proliferation in response to stimulation with ConA and PWM was similar in sRAGE-treated and control groups.

DISCUSSION

RAGE, a promiscuous pattern recognition receptor, binds a broad variety of pro-inflammatory ligands. Activation of cell-bound RAGE induces intracellular signal transduction followed by long-lasting NF-κB activation and inflammation (Bucciarelli et al. 2002; Schmidt et al. 2001). sRAGE functions as a “decoy” receptor competing with cell-bound RAGE for ligand binding and preventing ligands from interacting with receptors other than RAGE.

In previous experiments, we showed that sRAGE downregulated HMGB1-induced arthritis by redirecting leucocytes from the inflamed joints into the peritoneal cavity to the site of sRAGE injection (Pullerits et al. 2006). We concluded that sRAGE has chemotactic and pro-inflammatory properties (Pullerits et al. 2006). However, the exact mechanisms by which sRAGE regulates inflammatory responses and the effect of sRAGE on the immune cells in vivo are not well understood. We have therefore analysed the properties of sRAGE with respect to acquired immunity. In the present study, we provide evidence that sRAGE has immunomodulatory properties and induces a re-arrangement of cellular composition in lymphoid organs in naive mice.

Following intraperitoneal administration of sRAGE into healthy mice, we observed an increased influx of immune cells into the peritoneal cavity. The analysis of the cellular composition of the peritoneal fluid revealed an elevated number of B- and T-cells together with an increased population of Mac-1-expressing cells. Wang et al. (2010) recently demonstrated that intra-tracheal administration of sRAGE in healthy mice led to the activation of innate immunity and to an influx of monocytes and neutrophils into lung tissue resulting in lung inflammation and affecting immune homeostasis. Notably, the direct injection of sRAGE into murine lungs recruited more monocytes/macrophages than LPS at 24 h (Wang et al. 2010) indicating the prominent chemotactic properties of sRAGE. Additionally, sRAGE has been shown to induce chemotaxis in mouse neutrophils (Pullerits et al. 2006) and to recruit human neutrophils and monocytes in vitro (Wang et al. 2010). These results support our findings that sRAGE acts as a pro-inflammatory molecule and induces the recruitment of immune competent cells into the site of administration.

We observed that sRAGE treatment induced splenomegaly. Investigation of the cell distribution in the enlarged spleens revealed that the total number of B cells and the Mac-1+ macrophage population were significantly increased. Wang et al. (2010) recently demonstrated that treatment with sRAGE promoted human monocyte survival by protecting monocytes from apoptosis in a dose-dependent manner. The decreased apoptosis of macrophages could contribute to the increased cellularity in spleen found in our study. Simultaneously, reduced proportions of CD19+ B cells were seen in the bone marrow of sRAGE-treated mice suggesting that sRAGE treatment directs cells from the bone marrow towards the spleen.

Our unexpected finding that the frequency of Mac-1+ cells in the peritoneal cavity of sRAGE-treated mice decreased might have several reasons. First, as shown in our previous study (Pullerits et al. 2006) and confirmed by others (Frommhold et al. 2010), sRAGE can directly bind to the Mac-1 receptor that might interfere with the binding sites for the Mac-1 antibodies used for the detection of these cells by FACS. Second, sRAGE can mediate firm leucocyte adhesion in a Mac-1-dependent fashion and thereby activate leucocytes (Frommhold et al. 2010). These Mac-1+ cells undergoing conformational changes in response to integrin activation might be more difficult to detect, since receptor re-circulation is a well-known strategy of these cells (Mosser 1994).

Recent evidence indicates that sRAGE treatment modulates the differentiation of human monocytes into macrophages (Wang et al. 2010). In our study, spleen cells from sRAGE-treated mice showed increased IgM and IgG production compared with untreated mice. IgM is mostly produced by recently activated B cells, whereas B cells that have switched to a more mature class produce antibodies with IgG isotypes. These findings suggest that sRAGE leads to B cell activation and differentiation. Alternatively, because recently developed B cells leave the bone marrow following sRAGE administration, more mature B cells or plasmablasts might home back to the bone marrow to stabilize the altered homeostasis.

Our findings that sRAGE possibly acts as a potent B cell differentiation factor can further be supported by data showing that sRAGE treatment influences cytokine profile both in vivo and in vitro. Mice treated with sRAGE had significantly increased levels of IFN-γ. In addition, spleen cells from sRAGE-treated mice displayed a two-fold higher spontaneous production of IFN-γ in cell culture. Interferon-γ, which plays an important role in both innate and adaptive immunity, has been regarded traditionally as a pro-inflammatory cytokine but recent evidence suggests a bidirectional immune-regulatory role for this cytokine (Kelchtermans et al. 2008). IFN-γ acts as a principal activator of macrophages and B cells, in accordance with our findings, i.e. a higher number of IgG-producing B cells in the spleen and increased surface-bound IgG expression on CD19+ B cells in bone marrow. These data agree

with the significantly higher spontaneous in vitro production of IL-4 seen in spleen cells of sRAGE-treated mice. IL-4, produced mainly by CD4+ type 2 helper T cells, participates in the differentiation and growth of B cells and also in the differentiation of mature B cells into plasmablasts. Moreover, IL-6, a cytokine with prominent pro-inflammatory properties, stimulates the growth and differentiation of B-lymphocytes and increases significantly more in sRAGE-treated mice in response to LPS stimulation. Taken together, these observations indicate that sRAGE treatment in vivo modulates the cytokine profile and might favour B cell differentiation.

Interestingly, spleen cells from sRAGE-treated mice displayed a significantly increased production of IL-10 in response to LPS and ConA. IL-10 is an anti-inflammatory cytokine with a crucial role in preventing autoimmune and inflammatory pathologies (Saraiva and O'Garra 2010). IL-10 is produced not only by cells of the adaptive immune system including various T cell and B cell subsets, but also by macrophages (Saraiva and O'Garra 2010). The ability of B cells to produce IL-10 and thereby downregulate inflammation is thought to be associated with its regulatory role in experimental autoimmunity (Mauri and Blair 2010).

BAFF is a cytokine expressed predominantly by neutrophils, monocytes, macrophages and dendritic cells. BAFF is a regulator of B cell survival and acts in immunoglobulin isotype switch and B cell co stimulation (Mackay and Browning 2002). However, since we did not observe any differences in serum BAFF levels in sRAGE-treated mice compared with the controls, our results suggest that the sRAGE effect is not mediated via the BAFF pathway.

Surprisingly, we observed that sRAGE treatment in vivo reduces the capacity of splenocyte proliferation in vitro in response to LPS. One possible reason for this is that sRAGE might induce the maturation and differentiation of cells in vivo and that this overrides the proliferative signals caused by LPS stimulation in vitro. Indeed, Wang et al. (2010) showed that sRAGE interaction with monocytes induced the differentiation of these cells into macrophages, thus supporting our theory. Moreover, the high levels of IL-10 produced in splenocyte cultures in response to LPS stimulation might also have an inhibitory role on cell proliferation (Marcelletti 1996).

Taken together, our results indicate that sRAGE administration in naive mice leads to an influx of lymphocytes into the spleen, simultaneously reducing B-cell number in the bone marrow. Furthermore, sRAGE causes the activation and maturation of B cells resulting in a higher secretion of IgM and IgG and leads to the activation of T lymphocytes, as confirmed by the increased production of cytokines.

In conclusion, we show that sRAGE has a direct effect on the cells of adaptive immunity in naive mice. However, further studies are warranted to investigate in more detail the molecular mechanisms and to determine the B cell subsets that are involved in sRAGE-induced alterations of cellular distribution.

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REFERENCES

Amu S, Gjertsson I, Brisslert M (2010) Functional characterization of murine CD25 expressing B cells. Scand J Immunol 71:275-282

Amu S, Gjertsson I, Tarkowski A, Brisslert M (2006) B-cell CD25 expression in murine primary and secondary lymphoid tissue. Scand J Immunol 64:482-492

Brett J, Schmidt AM, Yan SD, Zou YS, Weidman E, Pinsky D, Nowygrod R, Neeper M, Przysiecki C, Shaw A, et al. (1993) Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. Am J Pathol 143:1699-1712

Bucciarelli LG, Wendt T, Rong L, Lalla E, Hofmann MA, Goova MT, Taguchi A, Yan SF, Yan SD, Stern DM, Schmidt AM (2002) RAGE is a multiligand receptor of the immunoglobulin superfamily: implications for homeostasis and chronic disease. Cell Mol Life Sci 59:1117-1128

Chavakis T, Bierhaus A, Al-Fakhri N, Schneider D, Witte S, Linn T, Nagashima M, Morser J, Arnold B, Preissner KT, Nawroth PP (2003) The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment. J Exp Med 198:1507-1515

Foell D, Wittkowski H, Roth J (2007a) Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. Nat Clin Pract Rheumatol 3:382-390

Foell D, Wittkowski H, Vogl T, Roth J (2007b) S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. J Leukoc Biol 81:28-37

Frommhold D, Kamphues A, Hepper I, Pruenster M, Lukic IK, Socher I, Zablotskaya V, Buschmann K, Lange-Sperandio B, Schymeinsky J, Ryschich E, Poeschl J, Kupatt C, Nawroth PP, Moser M, Walzog B, Bierhaus A, Sperandio M (2010) RAGE and ICAM-1 cooperate in mediating leukocyte recruitment during acute inflammation in vivo. Blood 116:841-849

Goova MT, Li J, Kislinger T, Qu W, Lu Y, Bucciarelli LG, Nowygrod S, Wolf BM, Caliste X, Yan SF, Stern DM, Schmidt AM (2001) Blockade of receptor for advanced glycation end-products restores effective wound healing in diabetic mice. Am J Pathol 159:513-525

Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, Avila C, Kambham N, Bierhaus A, Nawroth P, Neurath MF, Slattery T, Beach D, McClary J, Nagashima M, Morser J, Stern D, Schmidt AM (1999) RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell 97:889-901

Hofmann MA, Drury S, Hudson BI, Gleason MR, Qu W, Lu Y, Lalla E, Chitnis S, Monteiro J, Stickland MH, Bucciarelli LG, Moser B, Moxley G, Itescu S, Grant PJ, Gregersen PK, Stern DM, Schmidt AM (2002) RAGE and arthritis: the G82S polymorphism amplifies the inflammatory response. Genes Immun 3:123-135

Kalea AZ, Schmidt AM, Hudson BI (2012) Alternative splicing of RAGE: roles in biology and disease. Front Biosci 17:2756-2770

Kelchtermans H, Billiau A, Matthys P (2008) How interferon-gamma keeps autoimmune diseases in check. Trends Immunol 29:479-486

Liliensiek B, Weigand MA, Bierhaus A, Nicklas W, Kasper M, Hofer S, Plachky J, Grone HJ, Kurschus FC, Schmidt AM, Yan SD, Martin E, Schleicher E, Stern DM, Hammerling GG, Nawroth PP, Arnold B (2004) Receptor for advanced glycation end products (RAGE) regulates sepsis but not the adaptive immune response. J Clin Invest 113:1641-1650

Lotze MT, Tracey KJ (2005) High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nat Rev Immunol 5:331-342

Mauri C, Blair PA (2010) Regulatory B cells in autoimmunity: developments and controversies. Nat Rev Rheumatol 6:636-643

Mosser DM (1994) Receptors on phagocytic cells involved in microbial recognition. Immunol Ser 60:99-114

Park L, Raman KG, Lee KJ, Lu Y, Ferran LJ, Jr., Chow WS, Stern D, Schmidt AM (1998) Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. Nat Med 4:1025-1031

Pullerits R, Bokarewa M, Jonsson IM, Verdrengh M, Tarkowski A (2005) Extracellular cytochrome c, a mitochondrial apoptosis-related protein, induces arthritis. Rheumatology (Oxford) 44:32-39

Pullerits R, Brisslert M, Jonsson IM, Tarkowski A (2006) Soluble receptor for advanced glycation end products triggers a proinflammatory cytokine cascade via beta2 integrin Mac-1. Arthritis Rheum 54:3898-3907

Raucci A, Cugusi S, Antonelli A, Barabino SM, Monti L, Bierhaus A, Reiss K, Saftig P, Bianchi ME (2008) A soluble form of the receptor for advanced glycation endproducts (RAGE) is produced by proteolytic cleavage of the membrane-bound form by the sheddase a disintegrin and metalloprotease 10 (ADAM10). Faseb J 22:3716-3727

Saraiva M, O'Garra A (2010) The regulation of IL-10 production by immune cells. Nat Rev Immunol 10:170-181

Schmidt AM, Yan SD, Yan SF, Stern DM (2000) The biology of the receptor for advanced glycation end products and its ligands. Biochim Biophys Acta 1498:99-111

Schmidt AM, Yan SD, Yan SF, Stern DM (2001) The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. J Clin Invest 108:949-955

Wang Y, Wang H, Piper MG, McMaken S, Mo X, Opalek J, Schmidt AM, Marsh CB (2010) sRAGE induces human monocyte survival and differentiation. J Immunol 185:1822-1835

Vazzana N, Santilli F, Cuccurullo C, Davi G (2009) Soluble forms of RAGE in internal medicine. Intern Emerg Med 4:389-401

Yamagishi S, Matsui T (2010) Soluble form of a receptor for advanced glycation end products (sRAGE) as a biomarker. Front Biosci (Elite Ed) 2:1184-1195

Yan SS, Wu ZY, Zhang HP, Furtado G, Chen X, Yan SF, Schmidt AM, Brown C, Stern A, LaFaille J, Chess L, Stern DM, Jiang H (2003) Suppression of experimental autoimmune encephalomyelitis by selective blockade of encephalitogenic T-cell infiltration of the central nervous system. Nat Med 9:287-293

FIGURE LEGENDS

Figure 1

Soluble RAGE treatment causes influx of cells into peritoneal cavity and leads to splenomegaly.

Mice treated intraperitoneal with sRAGE display increased frequency (A) and total number of CD3, CD19 and Mac-1 expressing cells in the peritoneal lavage (B), have enlarged spleen (B) and increased cellularity in spleen (C) as compared with control mice injected with PBS.
Box plots show 25th and 75th percentiles; whiskers below and above vertical bars indicate 5th and 95th percentiles, respectively; solid lines within the boxes indicate medians. Statistical significance was tested using Mann-Whitney U test.

Figure 2

Soluble RAGE administration leads to changes in cellular composition in lymphoid organs.
Mice receiving intraperitoneal treatment with sRAGE display increased total number of CD19 and tendency towards increase of Mac-1 expressing cells in the spleen (A), whereas the number of CD19-positive cells is decreased in their bone marrow (B) as compared to vehicle-treated control group.
Box plots show 25th and 75th percentiles; whiskers below and above vertical bars indicate 5th and 95th percentiles, respectively. Statistical significance was tested using Mann-Whitney U test.

Figure 3

Soluble RAGE treatment leads to changes in cytokine profile
Mouse splenocytes from sRAGE treated (grey boxes) and control animals (white boxes) were stimulated with LPS, ConA, PWM or left un-stimulated (NS). Cell culture supernatants were collected for detection of IL-10, IFN-γ, IL-4 and IL-6 following 24 hours stimulation.
Box plots show 25th and 75th percentiles; whiskers below and above vertical bars indicate 5th and 95th percentiles, respectively. Statistical significance was tested using Mann-Whitney U test.

Figure 4

Spleen cells from sRAGE treated mice display increased antibody production.
Splenocytes obtained from mice following intraperitoneal sRAGE treatment display several fold increase in IgG and IgM antibodies as revealed by ELISPOT.
Two separate experiments were performed and the pooled results are reported as fold increase (mean ± SEM) from 9 mice per group.

Figure 5

Soluble RAGE alters the proliferative response to LPS.
Mouse splenocytes from sRAGE treated (grey boxes) and control animals (white boxes) were stimulated with LPS, ConA or left un-stimulated (NS). Lymphocytes from sRAGE treated animals display decreased proliferative response following stimulation with LPS.
Box plots show 25th and 75th percentiles; whiskers below and above vertical bars indicate 5th and 95th percentiles, respectively.

Figure 1.

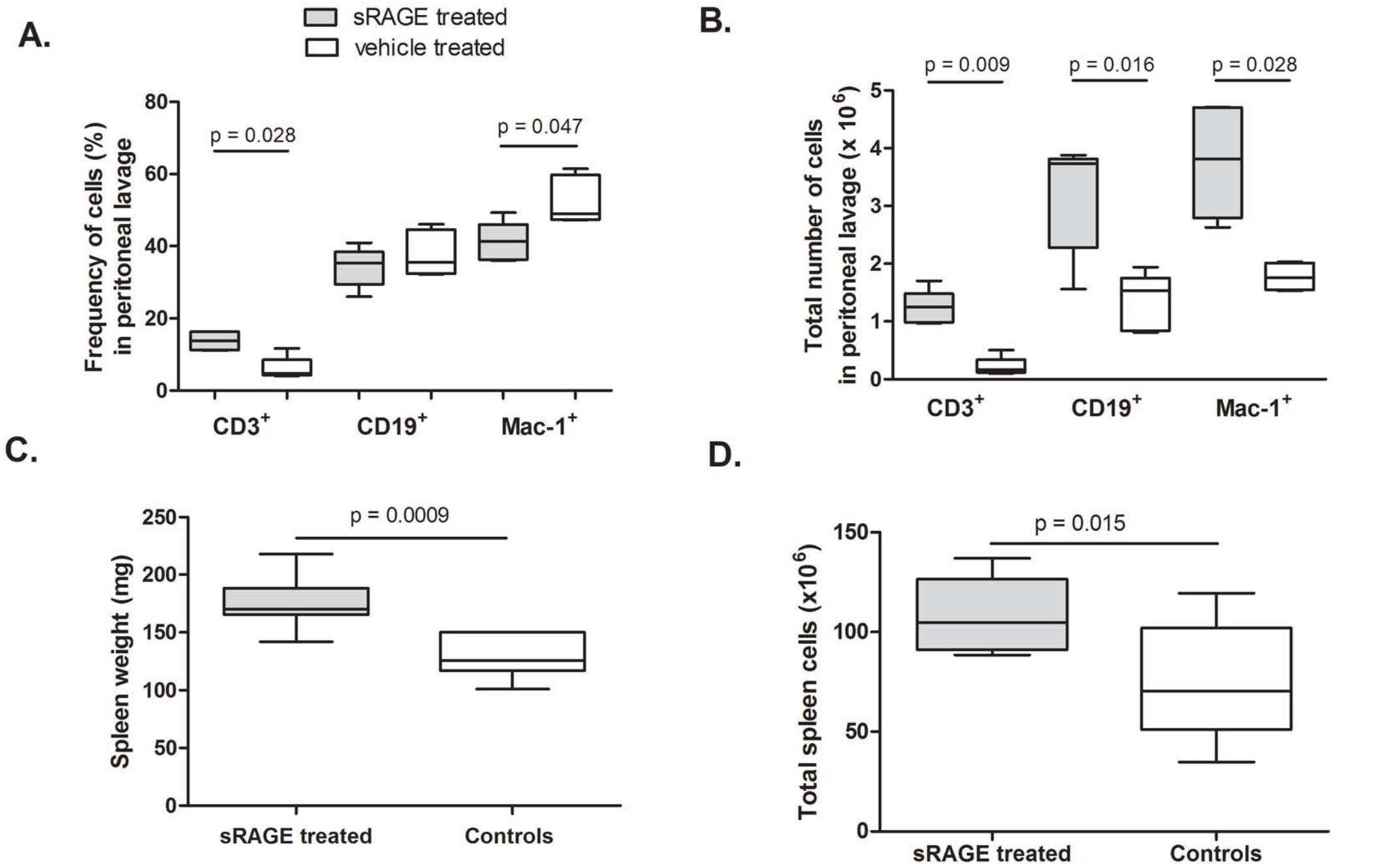

A.
sRAGE treated
vehicle treated
Frequency of cells (%) in peritoneal lavage
80
60
40
20
0
p = 0.028
p = 0.047
CD3+
CD19+
Mac-1+
B.
p = 0.009
p = 0.016
p = 0.028
Total number of cells in peritoneal lavage (x 10^6)
5
4
3
2
1
0
CD3+
CD19+
Mac-1+
C.
Spleen weight (mg)
250
200
150
100
50
0
p = 0.0009
sRAGE treated
Controls
D.
Total spleen cells (x10^6)
150
100
50
0
p = 0.015
sRAGE treated
Controls

Figure 2.

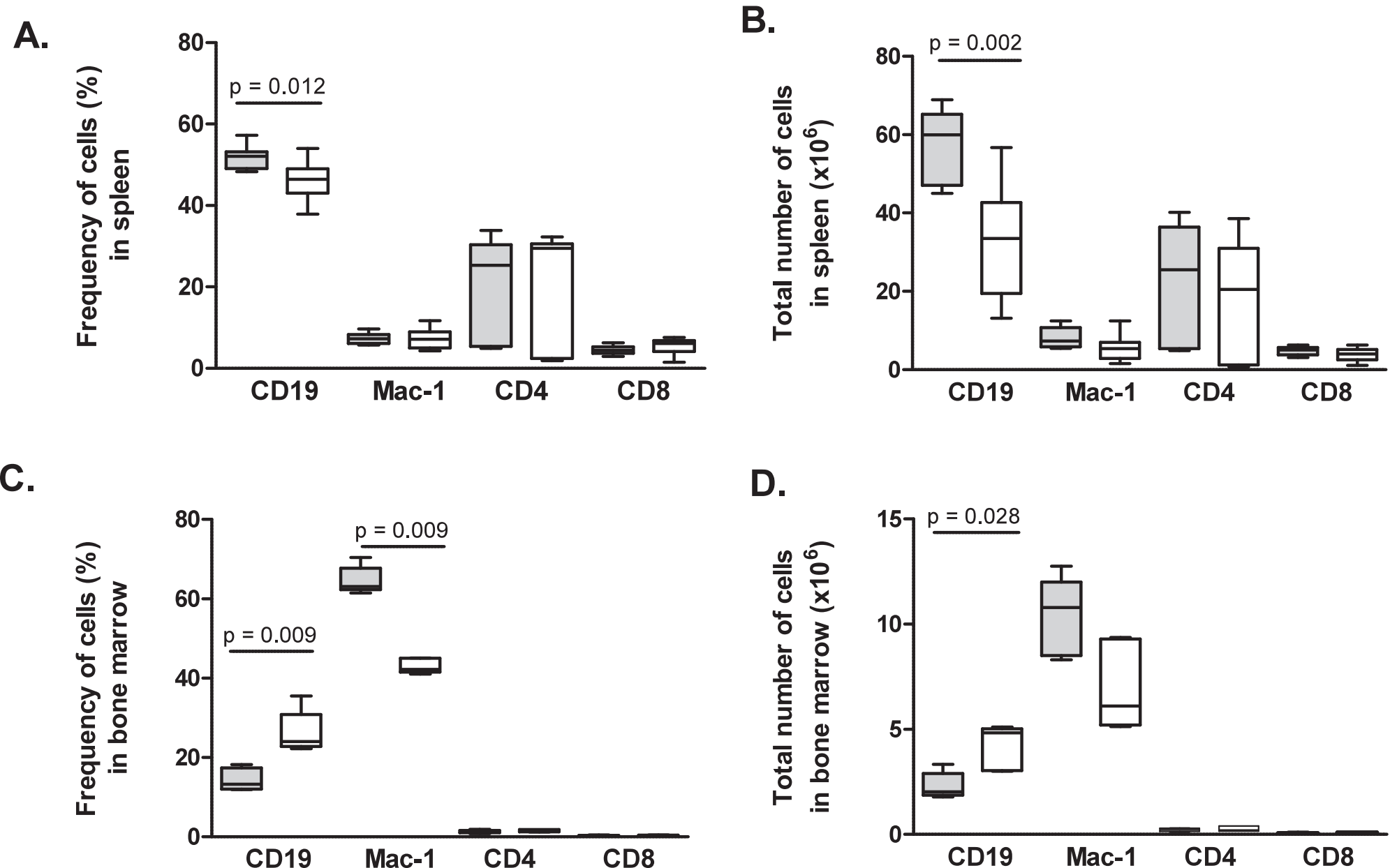
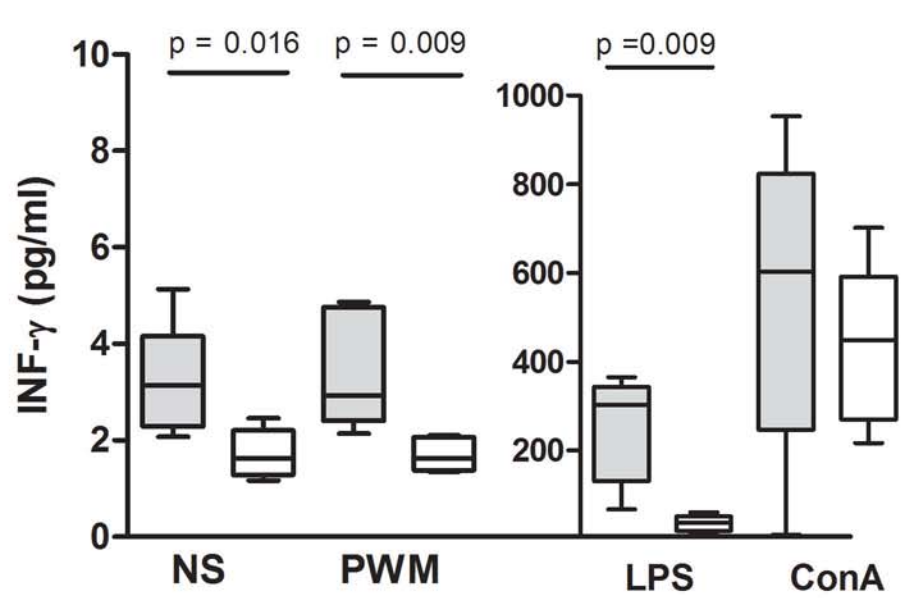
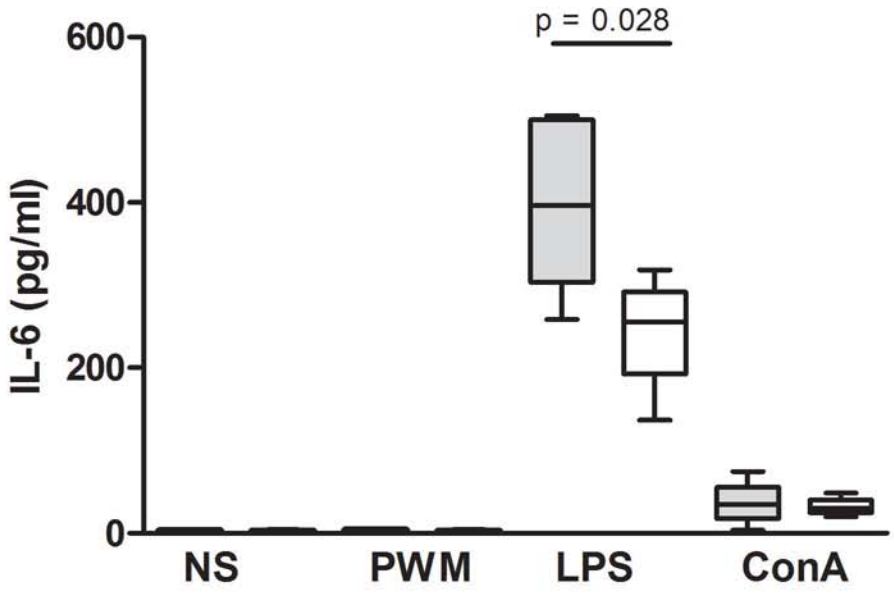
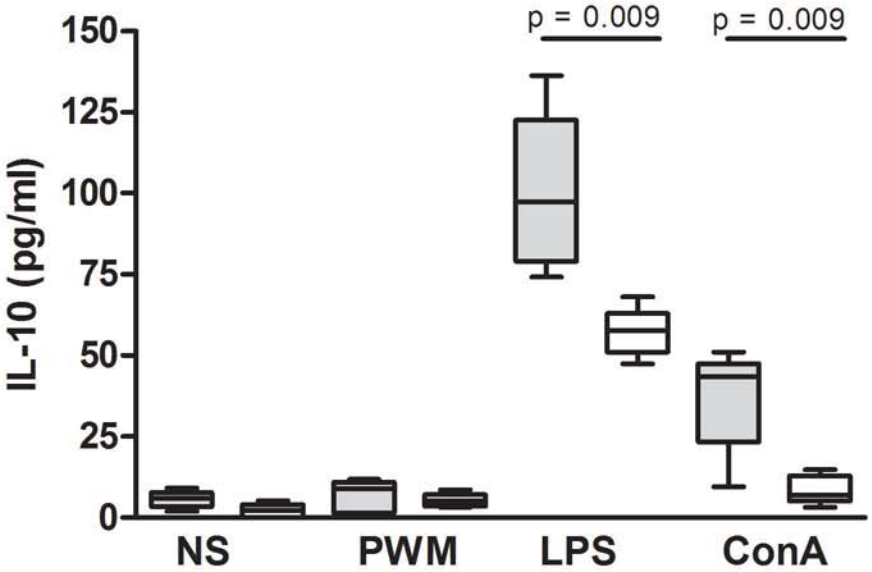

A.
Frequency of cells (%) in spleen
p = 0.012
80
60
40
20
0
CD19
Mac-1
CD4
CD8
B.
Total number of cells in spleen (x10^6)
p = 0.002
80
60
40
20
0
CD19
Mac-1
CD4
CD8
C.
Frequency of cells (%) in bone marrow
p = 0.009
p = 0.009
80
60
40
20
0
CD19
Mac-1
CD4
CD8
D.
Total number of cells in bone marrow (x10^6)
p = 0.028
15
10
5
0
CD19
Mac-1
CD4
CD8

Figure 3.


p = 0.016
p = 0.009
p =0.009
INF-γ (pg/ml)
10
8
6
4
2
0
1000
800
600
400
200
NS
PWM
LPS
ConA


p = 0.028
IL-6 (pg/ml)
600
400
200
0
NS
PWM
LPS
ConA


p = 0.009
p = 0.009
IL-10 (pg/ml)
150
125
100
75
50
25
0
NS
PWM
LPS
ConA

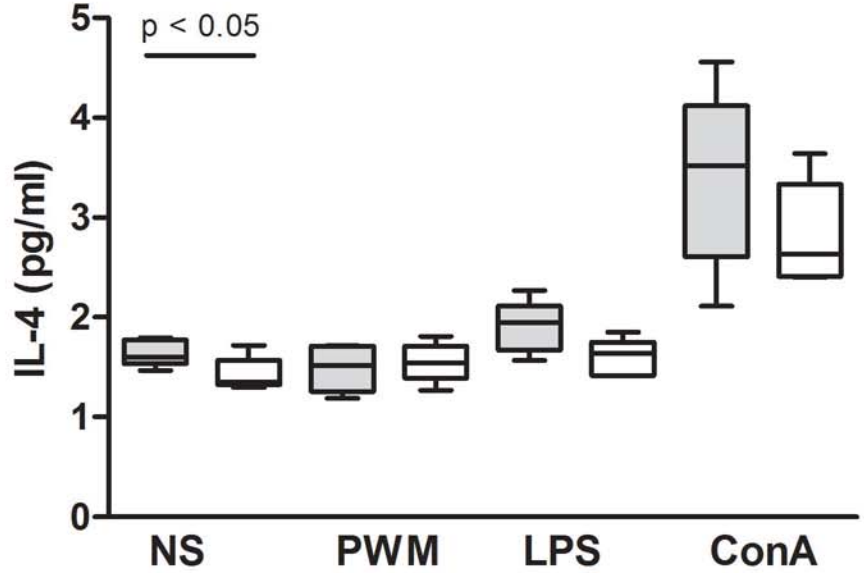

p < 0.05
IL-4 (pg/ml)
5
4
3
2
1
0
NS
PWM
LPS
ConA

Figure 4.

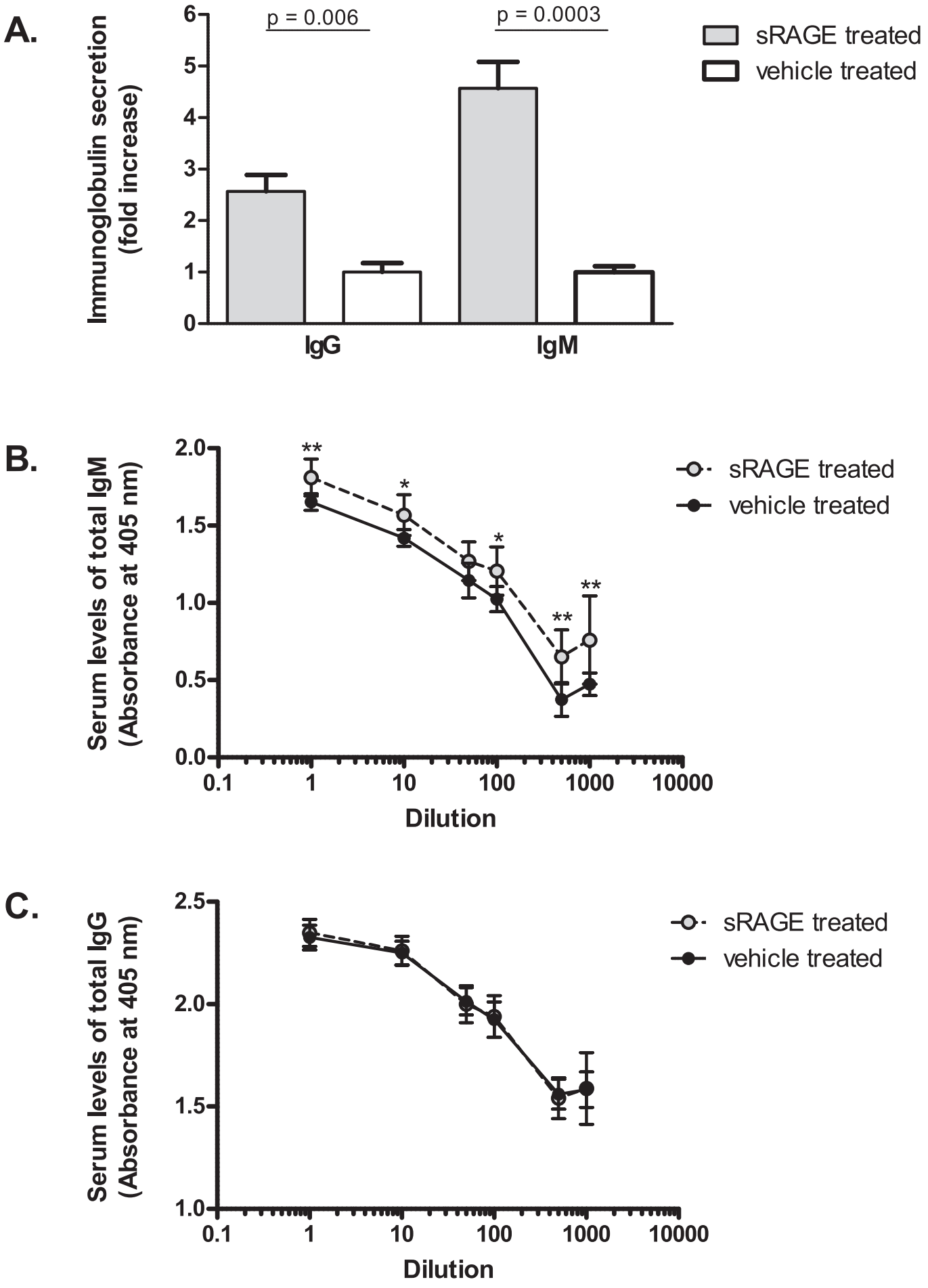
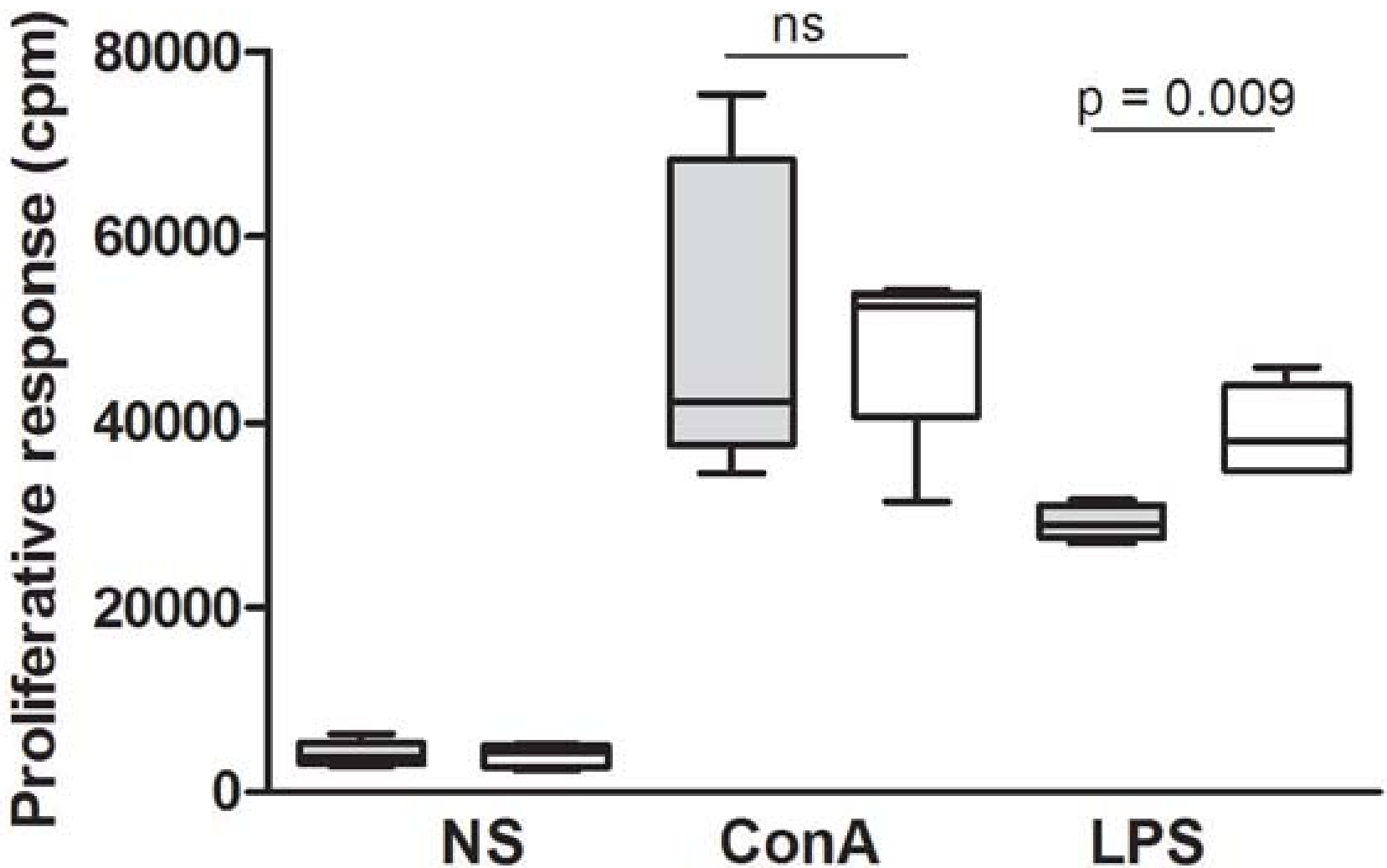

A.
p = 0.006
p = 0.0003
sRAGE treated
vehicle treated
Immunoglobulin secretion (fold increase)
0
1
2
3
4
5
6
IgG
IgM
B.
sRAGE treated
vehicle treated
Serum levels of total IgM (Absorbance at 405 nm)
0.0
0.5
1.0
1.5
2.0
**
*
*
**
**
0.1
1
10
100
1000
10000
Dilution
C.
sRAGE treated
vehicle treated
Serum levels of total IgG (Absorbance at 405 nm)
1.0
1.5
2.0
2.5
0.1
1
10
100
1000
10000
Dilution

Figure 5.


Proliferative response (cpm)
80000
60000
40000
20000
0
ns
p = 0.009
NS
ConA
LPS