A novel anti-inflammatory role for secretory phospholipase A$_2$ in immune complex-mediated arthritis

Eric Boilard[1], Ying Lai[2], Katherine Larabee[1], Barbara Balestrieri[1], Farideh Ghomashchi[2], Daisuke Fujioka[1], Reuben Gobezie[3], Jonathan S. Coblyn[1], Michael E. Weinblatt[1], Elena M. Massarotti[1], Thomas S. Thornhill[4], Maziar Divangahi[1], Heinz Remold[1], Gérard Lambeau[5], Michael H. Gelb[2], Jonathan P. Arm[1]**, David M. Lee[1]*

Phospholipase A$_2$ (PLA$_2$) catalyses the release of arachidonic acid for generation of lipid mediators of inflammation and is crucial in diverse inflammatory processes. The functions of the secretory PLA$_2$ enzymes (sPLA$_2$), numbering nine members in humans, are poorly understood, though they have been shown to participate in lipid mediator generation and the associated inflammation. To further understand the roles of sPLA$_2$ in disease, we quantified the expression of these enzymes in the synovial fluid in rheumatoid arthritis and used gene-deleted mice to examine their contribution in a mouse model of autoimmune erosive inflammatory arthritis. Contrary to expectation, we find that the group V sPLA$_2$ isoform plays a novel anti-inflammatory role that opposes the pro-inflammatory activity of group IIA sPLA$_2$. Mechanistically, group V sPLA$_2$ counter-regulation includes promotion of immune complex clearance by regulating cysteinyl leukotriene synthesis. These observations identify a novel anti-inflammatory function for a PLA$_2$ and identify group V sPLA$_2$ as a potential biotherapeutic for treatment of immune-complex-mediated inflammation.

INTRODUCTION

Phospholipase A$_2$ (PLA$_2$) comprise a diverse family whose members share the capacity to hydrolyse the *sn*-2 position of membrane glycerophospholipids, releasing fatty acids and lysophospholipids. There are over 25 mammalian PLA$_2$ isoforms that have been grouped into three major classes, namely the calcium-dependent and -independent intracellular enzymes, and calcium-dependent secreted PLA$_2$ (sPLA$_2$) (Schaloske & Dennis, 2006; Valentin & Lambeau, 2000). The best characterized member of the PLA$_2$ family is cytosolic group IV PLA$_2\alpha$, which is constitutively expressed in most tissues. Deletion of group IV cPLA$_2\alpha$ confirms its essential role in parturition and fertility (Bonventre et al, 1997; Kudo & Murakami, 2002; Uozumi et al, 1997) and its contributions to diverse inflammatory processes (Hegen et al, 2003, 2006).

The sPLA$_2$ family members are strikingly diverse. They are typically $\sim$14–19 kDa heavily disulphide bridged proteins found

not only in mammals, but also in insects, snake venoms, plants, bacteria, fungi and viruses (Kini, 2003; Lambeau & Gelb, 2008; Nagiec et al, 2004; Soragni et al, 2001; Zadori et al, 2001). Sequence homology analysis led to the identification of 10 mammalian enzymatically active sPLA$_2$s (nine of which are expressed in humans) and two sPLA$_2$-like proteins devoid of catalytic activity.

sPLA$_2$ enzymes have been implicated in physiological functions, host defence and inflammation. Group IB sPLA$_2$ is present in pancreatic secretions and exhibits a role in dietary phospholipid digestion (Arnesjo et al, 1967; Huggins et al, 2002). Group IIA sPLA$_2$ demonstrates a predilection for negatively charged membranes such as those present in bacterial cell walls and has been implicated in host defence against Gram-positive bacteria (Foreman-Wykert et al, 1999; Menschikowski et al, 2006; Piris-Gimenez et al, 2005). In a mechanistically distinct manner, group V sPLA$_2$ also plays a role in host defence by promoting phagocytosis and killing of fungal species (Balestrieri et al, 2006, 2009).

sPLA$_2$ activity has been demonstrated in multiple inflammatory disease states including rheumatoid arthritis (RA), sepsis, psoriasis, pancreatitis and cancer (Funakoshi et al, 1993; Green et al, 1991; Mounier et al, 2008; Pruzanski et al, 1985). The majority of these studies have focussed on the role of group IIA sPLA$_2$ in these processes. However, many of these studies were performed prior to the characterization of the multiple sPLA$_2$ isoforms and before the development of isoform-specific reagents. Recent descriptions of the pro-inflammatory role of group V sPLA$_2$ in allergic airway inflammation (Munoz et al, 2007), acute lung injury (Munoz et al, 2009) and atherosclerosis (Bostrom et al, 2007) prompted us to re-examine the presence of sPLA$_2$ isoforms

in RA synovial fluid (SF) and to evaluate the role of group V sPLA$_2$ in arthritis. Surprisingly, our data indicate a counter-regulatory role for group V sPLA$_2$ in a mouse model of immune complex-mediated arthritis. Further, we delineate a novel mechanism, shared in mice and humans, in which group V sPLA$_2$ promotes the phagocytosis of immune complexes by macrophages to ameliorate inflammation. This group V sPLA$_2$ function depends on its enzymatic activity and the generation of cysteinyl leukotrienes (cysLTs). Interestingly, we also confirm the pro-inflammatory role of group IIA sPLA$_2$ in arthritis.

These findings highlight the complexity of sPLA$_2$ species' participation in inflammation, reveal a previously unappreciated and unanticipated anti-inflammatory function for group V sPLA$_2$, underscore the importance of developing selective inhibitors of pro-inflammatory sPLA$_2$ members for use in therapy and identify group V sPLA$_2$ as a novel potential biotherapeutic in arthritis.

RESULTS

Diverse sPLA$_2$ are detected in RA synovial fluids

Given recent insights into distinct functional activities for sPLA$_2$ species, and since most analyses of PLA$_2$ enzymatic activity in SF were performed prior to cloning the complete family of sPLA$_2$ isoforms (Pruzanski et al, 1992, 1985; Seilhamer et al, 1989a; Vadas et al, 1985), we quantified the concentration of all catalytically active human sPLA$_2$ in SF from 45 subjects with RA using a specific immunoassay that distinguishes the different sPLA$_2$ isoforms (Fig 1A)
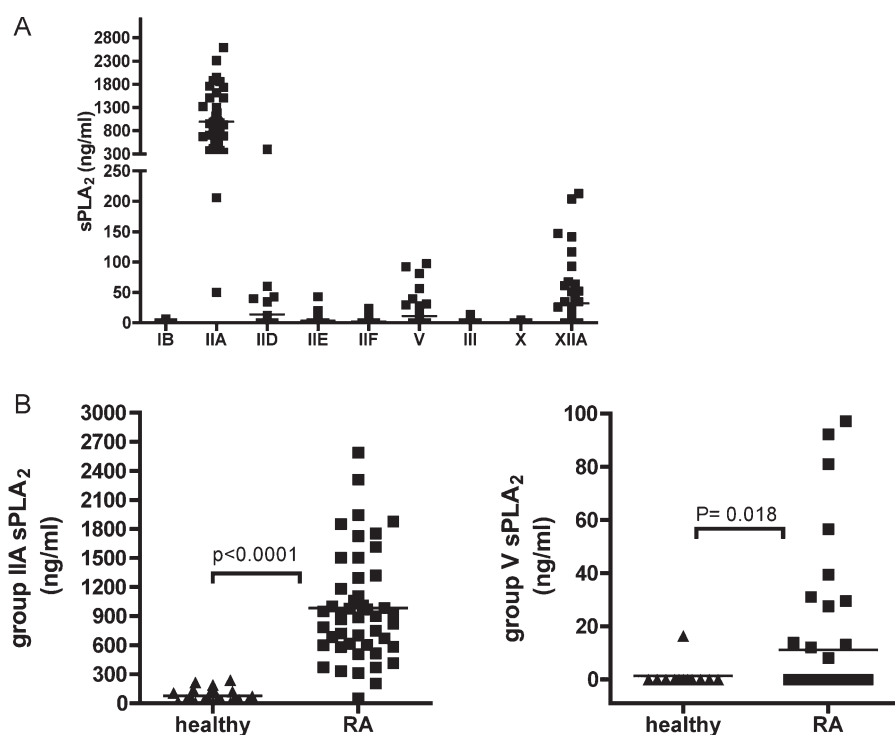

Figure 1. Expression of sPLA$_2$ in the SF of patients with RA and healthy controls.

A. The full set of human sPLA$_2$ isoforms were quantified by time-resolved immunofluorescence analysis in SF from patients with RA ($n = 45$).

B. Groups IIA and V sPLA$_2$ concentrations in SF obtained from healthy volunteers ($n = 12$).
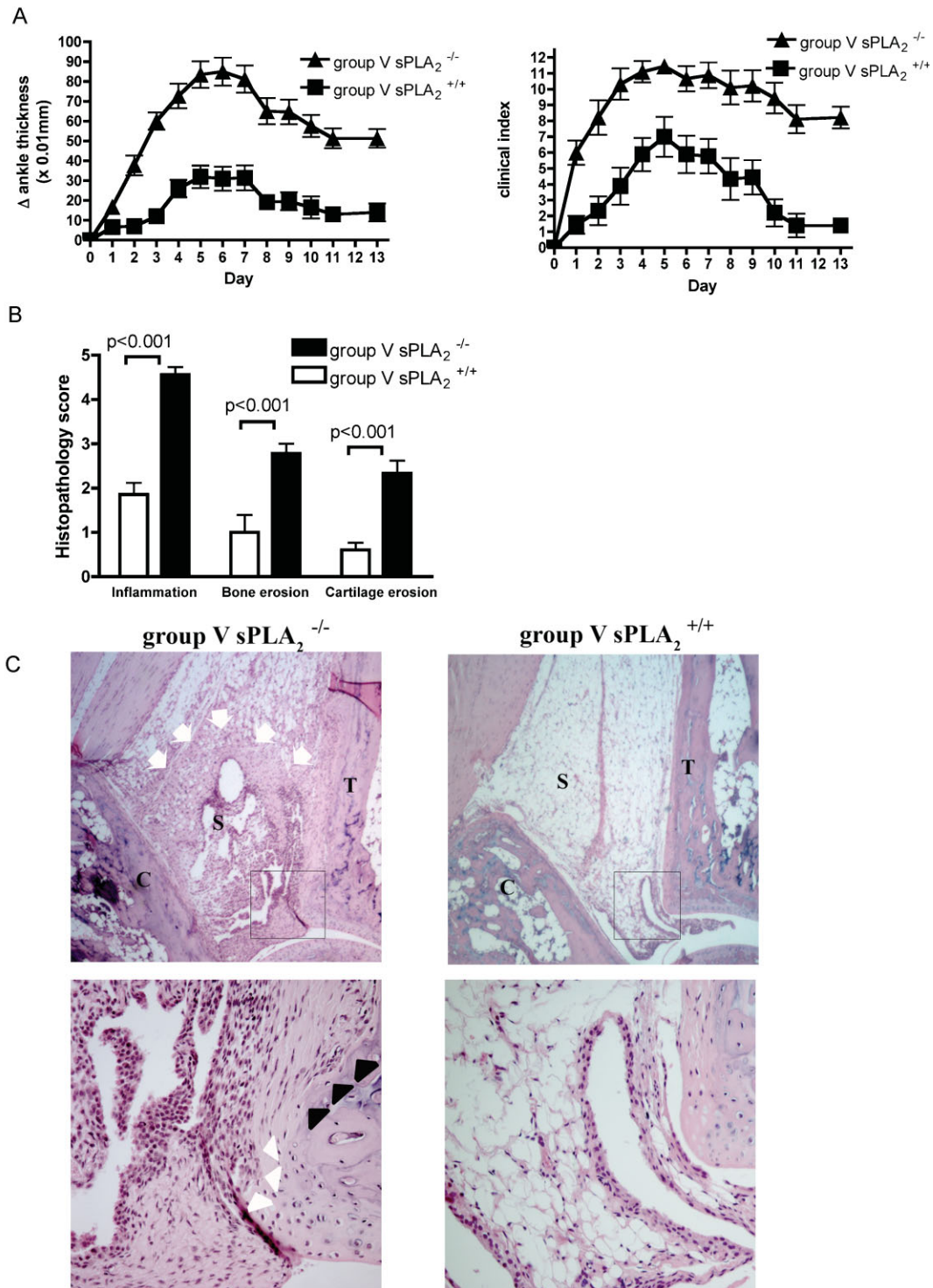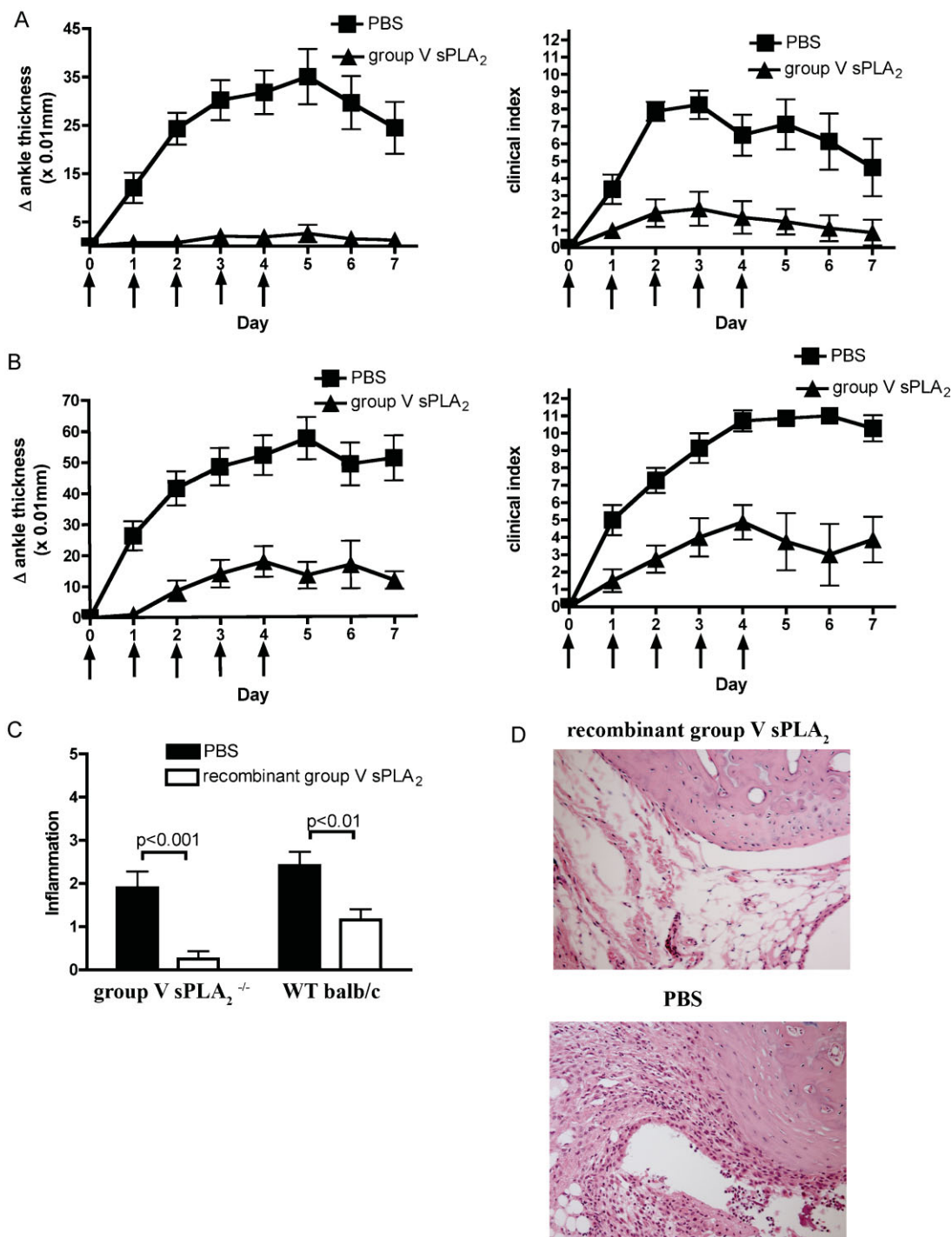
Figure 2. Group V sPLA$_2$ protects from K/BxN serum-transfer arthritis.

A. Arthritis response in group V sPLA$_2$ null and congenic control mice in a BALB/c group IIA null background. Mice were injected with 20 µl of K/BxN serum at days 0 and 2, and disease development was monitored for 13 days.

B. Histomorphometric quantification of arthritis severity in group V sPLA$_2$ null and congenic control mice at experimental day 13. $N = 15$ mice/group. Data are mean $\pm$ SEM pooled from three independent experiments. $P < 0.001$ for (A).

C. Representative mid-saggital ankle sections from group V sPLA$_2$ null and group V sPLA$_2$ control mice. Upper and lower panels are 25$\times$ and 200$\times$ magnification, respectively (lower panel). White arrows demarcate the hyperplastic synovial lining surrounding a large effusion (upper) while black and white arrowheads highlight bone and cartilage erosions, respectively. Note the increased leukocytic infiltration, synovial lining hyperplasia and pannus formation in group V sPLA$_2$ null mice (T, tibia; S, synovial space; C, calcaneus). Figures representative of findings in 15 mice/group.

(Nevalainen et al, 2005). Interestingly, all sPLA$_2$ isoforms could be detected, although typically each isoform was expressed in only a subgroup of subjects.

To examine disease-associated differences in sPLA$_2$ isoform expression, we also quantified sPLA$_2$ levels in SF from healthy volunteers. Here, due to limitations in SF volumes obtained from healthy individuals, we confined our analyses to groups IIA and V sPLA$_2$. Group IIA sPLA$_2$ was more prominently expressed than group V sPLA$_2$ in both healthy and RA subjects, and both isoforms were present in SF of RA subjects at levels significantly higher than observed in SF of healthy volunteers (Fig 1B). Examination of co-expression of groups IIA and V sPLA$_2$ demonstrates no statistically significant correlation between isoform levels in RA SF ($r = 0.3497$, $P = 0.1326$),

suggesting that the expression of the two enzymes is independently regulated.

Anti-inflammatory activity of group V sPLA$_2$ in autoimmune arthritis

Given the failed clinical trial of a putative group IIA sPLA$_2$ inhibitor in arthritis (Bradley et al, 2005) and having demonstrated expression of group V sPLA$_2$ in arthritic human SF, we examined the contribution of group V sPLA$_2$ to inflammatory arthritis. We employed the K/BxN serum-transfer model of autoimmune inflammatory arthritis to explore the contributions of group V sPLA$_2$ to the pathophysiology of inflammatory arthritis *in vivo*. The progressive distal symmetric erosive polyarthritis observed in K/BxN T-cell receptor (TcR) transgenic mice results from recognition of an ubiquitous autoantigen, glucose-6-phosphate isomerase (GPI), presented by the MHC class II A^{g7} molecule (Korganow et al, 1999; Kouskoff et al, 1996; Matsumoto et al, 1999). These autoreactive T-cells drive high titre pathogenic autoantibody production. Importantly, arthritis can be induced in recipient mice by passive transfer of arthritogenic IgG autoantibodies (Korganow et al, 1999; Matsumoto et al, 2002). Numerous IgG-driven effector phase mechanisms have been identified in the pathophysiology of this arthritis including multiple innate cellular lineages and soluble mediators (IL-1β, TNF, complement C5a/C5aR, eicosanoids (LTB$_4$ and PGI$_2$) and tryptase) (Boilard et al, 2010; Bruhns et al, 2003; Chen et al, 2006, 2008; Chiba et al, 2005; Corr & Crain, 2002; Ji et al, 2002a,b; Kim et al, 2006; Lee et al, 2002; Shin et al, 2009; Wipke & Allen, 2001). Of particular relevance, since the pathophysiology of the arthritis in the K/BxN serum-transfer model includes a contribution from inflammatory eicosanoids (Chen et al, 2006, 2008), it provides an ideal model in which to investigate the role of enzymes involved in eicosanoid biosynthesis.

To assess the *in vivo* role of group V sPLA$_2$ in inflammatory arthritis, we examined the response of group V sPLA$_2$ null and congenic control mice to administration of arthritogenic K/BxN serum. Unexpectedly, rather than showing attenuation of the arthritic response, mice that lack group V sPLA$_2$ demonstrated a significantly more severe autoantibody-driven arthritic response than congenic controls (Fig 2A). Histomorphometric analyses confirm clinical measures of arthritis, with increased leukocytic tissue infiltration, pannus formation and bone and cartilage destruction in group V sPLA$_2$ null mice (Fig 2B and C).

Systemic administration of recombinant group V sPLA$_2$ ameliorates arthritis

To validate the modulating role of endogenous group V sPLA$_2$ in antibody-driven inflammatory arthritis and to provide *in vivo* proof of concept for therapeutic use of group V sPLA$_2$, we produced highly purified recombinant mouse group V sPLA$_2$ and administered this material parenterally to group V sPLA$_2$ null mice. Mice deficient in group V sPLA$_2$ treated with recombinant group V sPLA$_2$ were substantially protected from K/BxN arthritis (Fig 3A, C and D). Interestingly, parenteral administration of recombinant group V sPLA$_2$ to wild-type (WT) mice also resulted in reduced clinical and histomorphometric indices of arthritis (Fig 3B and C). Taken together, these results confirm the counter-regulatory role for group V sPLA$_2$ in inflammatory arthritis that was observed in the genetic studies.

Toxicity considerations comprise an important aspect in evaluating both mechanistic activity and therapeutic potential for a novel disease target. Although little is known regarding potential toxic or deleterious activities for group V sPLA$_2$, previous studies in group V sPLA$_2$ transgenic mice have shown that transgenic neonatal pups die from pulmonary distress within 8 h after birth due to surfactant hydrolysis (Ohtsuki et al, 2006). To assess this potential confounder for our mechanistic and biotherapeutic studies, we examined lung tissues from mice administered recombinant group V sPLA$_2$ and found no evidence for tissue abnormality (Supporting Information Fig S1).

Group IIA sPLA$_2$ contributes to synovial inflammation

Since the anti-inflammatory activity of group V sPLA$_2$ was unexpected, we expanded our analyses by investigating whether group IIA sPLA$_2$ displayed an expected pro-inflammatory role in disease. As anticipated, mice with an isolated deficiency in group IIA sPLA$_2$ displayed substantial reduction of clinical signs of arthritis relative to congenic WT mice (Fig 4A and B). Histomorphometric quantification of tissue pathology confirmed clinical measures of arthritis, with decreases in leukocytic infiltration, bone erosion and cartilage destruction by synovial pannus in group IIA sPLA$_2$ null mice (Fig 4D).

Although most closely related by sequence homology (Seilhamer et al, 1989b), whether human group IIA sPLA$_2$ is the functional orthologue of murine group IIA sPLA$_2$ remains

Figure 3. Systemic administration of recombinant group V sPLA$_2$ ameliorates K/BxN serum-transfer arthritis.

A. Recombinant mouse group V sPLA$_2$ (50 μg in PBS) or control PBS were injected intravenously into group V sPLA$_2$-null mice in a BALB/c group IIA null background 2 hours prior to administration of K/BxN serum on experimental Day 0 and daily thereafter for 5 days. Arthritis was induced by injection of 35 μl and 20 μl of K/BxN serum on day 0 and 2 respectively; the development of arthritis was monitored for 7 days. Arrows indicate sPLA$_2$-V intravenous injections.

B. Experiment as detailed in (A) using WT BALB/c mice

C. Histomorphometric quantification of inflammation in ankle sections from mice injected with recombinant group V sPLA$_2$ or PBS control at experimental day 7. Note that the BALB/c mice express both group V and group IIA sPLA$_2$ and therefore are not the congenic controls to group V sPLA$_2$$^{-/-}$. $N = 12$ mice/group. Data are mean ± SEM pooled from three independent experiments. $P < 0.001$ for (A and B).

D. Representative mid-saggital ankle sections from group V sPLA$_2$ null mice treated with recombinant group V sPLA$_2$ or its diluent (PBS). Note the decreased leukocytic infiltration, synovial lining hyperplasia and pannus formation in recombinant group V sPLA$_2$ treated mice. Magnification = 200×. Figures are representative of findings in 12 mice/group.
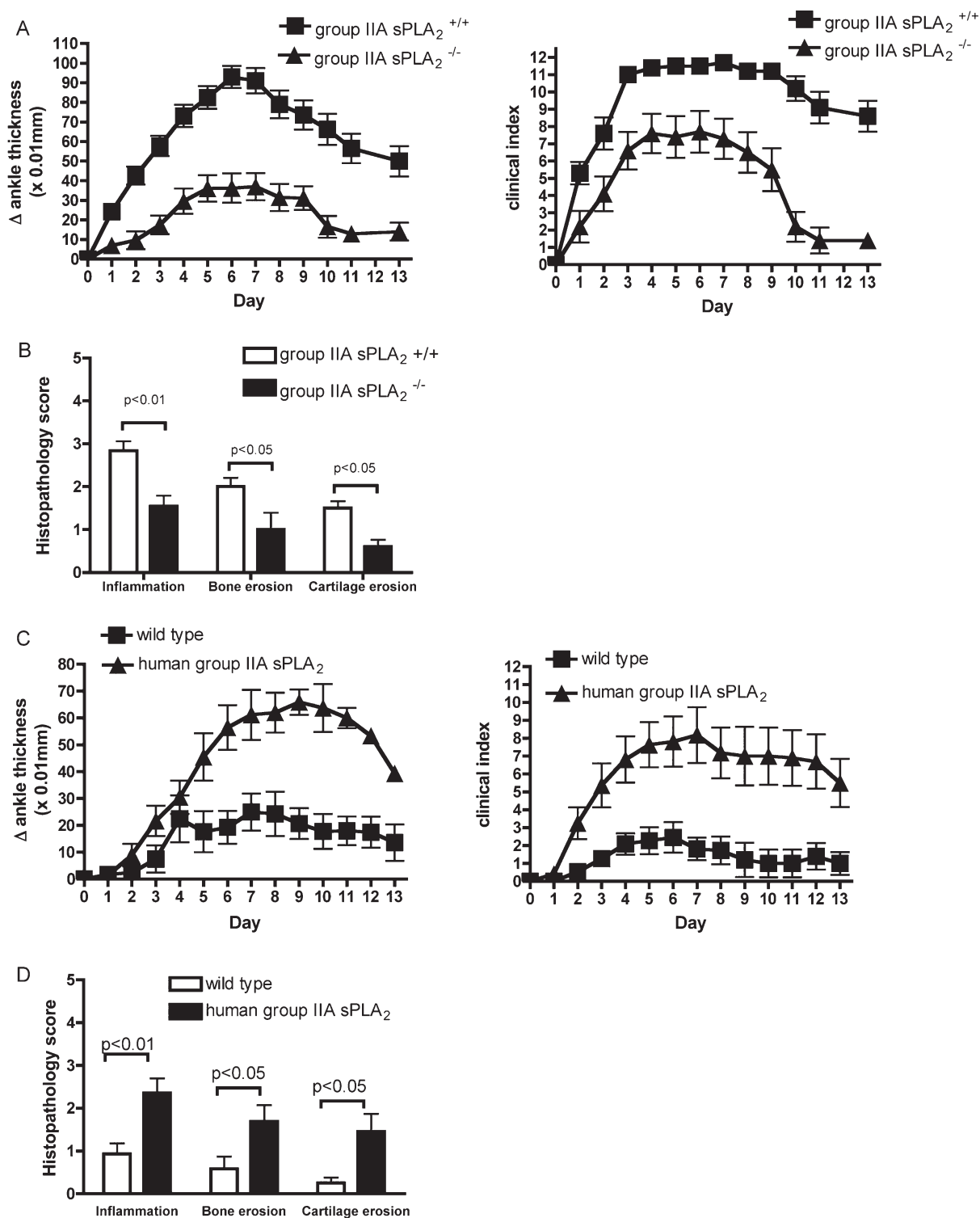
Figure 4. Group IIA sPLA$_2$ contributes to severity of K/BxN serum-transfer arthritis. Mice were injected with 65 and 35 μl K/BxN serum on days 0 and 2, respectively, and the development of arthritis was followed for 13 days.

A. Arthritic response in group IIA sPLA$_2$ null and WT congenic BALB/c control mice.

B. Histomorphometric quantification of arthritis severity in group IIA sPLA$_2$ null and congenic BALB/c control mice at experimental day 13. $N = 15$ mice/group. Data are mean $\pm$ SEM pooled from three independent experiments. $P < 0.001$ (A).

C. Human group IIA sPLA$_2$ transgenic and WT C57BL/6 mice were administered a single 75 μl dose of K/BxN serum on experimental day 0 and development of arthritis was monitored for 13 days.

D. Histomorphometric quantification of arthritis severity in human group IIA sPLA$_2$ transgenic and WT control mice at experimental day 13. $N = 15$ mice/group. Data are mean $\pm$ SEM pooled from three independent experiments. $P < 0.001$ for (C).

speculative. To confirm the pro-inflammatory contribution of human group IIA sPLA$_2$ to synovitis, we assessed the severity of K/BxN arthritis in mice expressing a human group IIA sPLA$_2$ transgene (Grass et al, 1996). Because the C57BL/6 (B6) strain contains a spontaneous mutation in group IIA sPLA$_2$ that abrogates expression (Kennedy et al, 1995), we selected human group IIA sPLA$_2$ transgenic mice on this background. Thus, the only group IIA sPLA$_2$ activity in these mice derives from the human transgene. Consistent with a pro-inflammatory contribution from group IIA sPLA$_2$ to human autoimmune arthritis, group IIA sPLA$_2$ transgenic mice display increased clinical (Fig 4C) and histological (Fig 4D) arthritic responses to K/BxN serum transfer.

Group X sPLA$_2$ deficiency does not impact arthritis

Group V sPLA$_2$ and group X sPLA$_2$ are unique among mammalian sPLA$_2$s in that they bind with high affinity to phosphatidylcholine-rich membranes and readily hydrolyse the external leaflet of mammalian cell membranes (Singer et al, 2002). We therefore examined arthritic responses in mice lacking group X sPLA$_2$. We found no contribution of group X sPLA$_2$ in K/BxN serum-induced erosive arthritis (Supporting Information Fig S2), further confirming separate and distinct functions of individual sPLA$_2$ enzymes in arthritis pathophysiology.

Group V sPLA$_2$ stimulates phagocytic uptake of immune complexes

Several of the known activities of group V sPLA$_2$ activities could plausibly contribute to its impact on inflammatory arthritis. We have previously demonstrated reduced phagocytosis of fungal particles and IgG-coated sheep red blood cells (RBC) in group V sPLA$_2$ deficient macrophages (Balestrieri et al, 2006, 2009). We therefore hypothesized that group V sPLA$_2$-directed phagocytosis of immune complexes by macrophages or other phagocytes could comprise a novel mechanism by which this isoform modulates arthritis activity. We thus monitored immune complex phagocytosis by primary murine macrophages and found impaired immune complex uptake into cells lacking group V sPLA$_2$ (Fig 5A).

To assess a homologous activity for human group V sPLA$_2$ and thereby extend the relevance of our observations to human autoimmune inflammatory disease, we added exogenous human group V sPLA$_2$ to leukocytes present in SF from inflamed joints of RA patients and monitored phagocytosis of IgG immune complexes by CD14+ macrophages. Consistent with our murine observations, we found that human group V sPLA$_2$ can trigger phagocytosis of IgG immune complexes in this population of cells that are abundant in the diseased joint fluid (Fig 5B).

Group V sPLA$_2$ promotes immune complex phagocytosis via cysteinyl leukotriene generation

We further examined the mechanisms by which group V sPLA$_2$ promotes phagocytic uptake of IgG-containing immune complexes. Previous studies have demonstrated that group V sPLA$_2$ activities include both phospholipase enzymatic activity and capacity to interact with the M-type receptor (Rouault et al, 2007). To define a contribution via its enzymatic activity, we mutated the catalytic site of group V sPLA$_2$ to generate an enzymatically inactive protein (group V sPLA$_2$-H48Q) (Lambeau & Gelb, 2008). We found this catalytically inactive mutant incapable of stimulating phagocytosis of IgG immune complexes by human SF CD14+ monocyte/macrophage cells (Fig 5B).

Having demonstrated a requirement for phospholipid hydrolysis by group V sPLA$_2$, we endeavoured to identify which lipid(s) promotes immune complex phagocytosis. Once released from the phospholipidic bilayer by a PLA$_2$, arachidonic acid can be metabolized into several classes of biologically active lipids including prostanoids, leukotrienes, lipoxins, resolvins and others via the cyclooxygenase (COX) or lipoxygenase (LO) pathways. Using pharmacologic inhibitors, we found that inhibition of 5-LO, but not COX, abrogated the promotion of immune complex phagocytosis by group V sPLA$_2$ in human SF CD14+ monocyte/macrophage cells (Fig 5C).

To define which 5-LO-dependent eicosanoid mediates group V sPLA$_2$ activity, we next utilized a candidate-based approach. Resolvins and lipoxins have documented anti-inflammatory activities (Haworth et al, 2008; Schwab & Serhan, 2006; Schwab et al, 2007), however, neither lipoxin A4 nor resolvin E1 promoted phagocytosis of immune complexes by macrophages when added exogenously (Supporting Information Fig S3A and B). Since we previously observed an unexplained increase in the severity of arthritis in mice lacking cysLTs (Chen et al, 2006) we investigated a role for cysLTs in the ability of group V sPLA$_2$ to promote immune complex uptake by phagocytes. Examination of supernatants from RA SF leukocytes or peripheral blood mononuclear cells (PBMC) treated with group V sPLA$_2$ demonstrated significant stimulation of cysLT generation by these populations (Figs 5D and 6D). In stark contrast, group IIA sPLA$_2$ and enzymatically inactive group V sPLA$_2$-H48Q lacked the ability to drive cysLT production in these cells (Figs 5D and 6D). Confirming studies by others (Mancuso & Peters-Golden, 2000), we found that both LTC$_4$ and LTD$_4$ potently promote immune complex clearance when added exogenously to these cell populations (Fig 5E and F and Supporting Information Fig S4). In further confirmation, we found that administration of a cysteinyl leukotriene receptor 1 (CysLTR1) antagonist inhibits promotion by group V sPLA$_2$ of immune complex phagocytosis by CD14+ cells (Fig 5G). In sum, our findings point to a novel pathway in which group V sPLA$_2$ promotes immune complex clearance in monocyte/macrophage cells via stimulating synthesis of CysLTs, which act through CysLTR1 to promote phagocytosis.
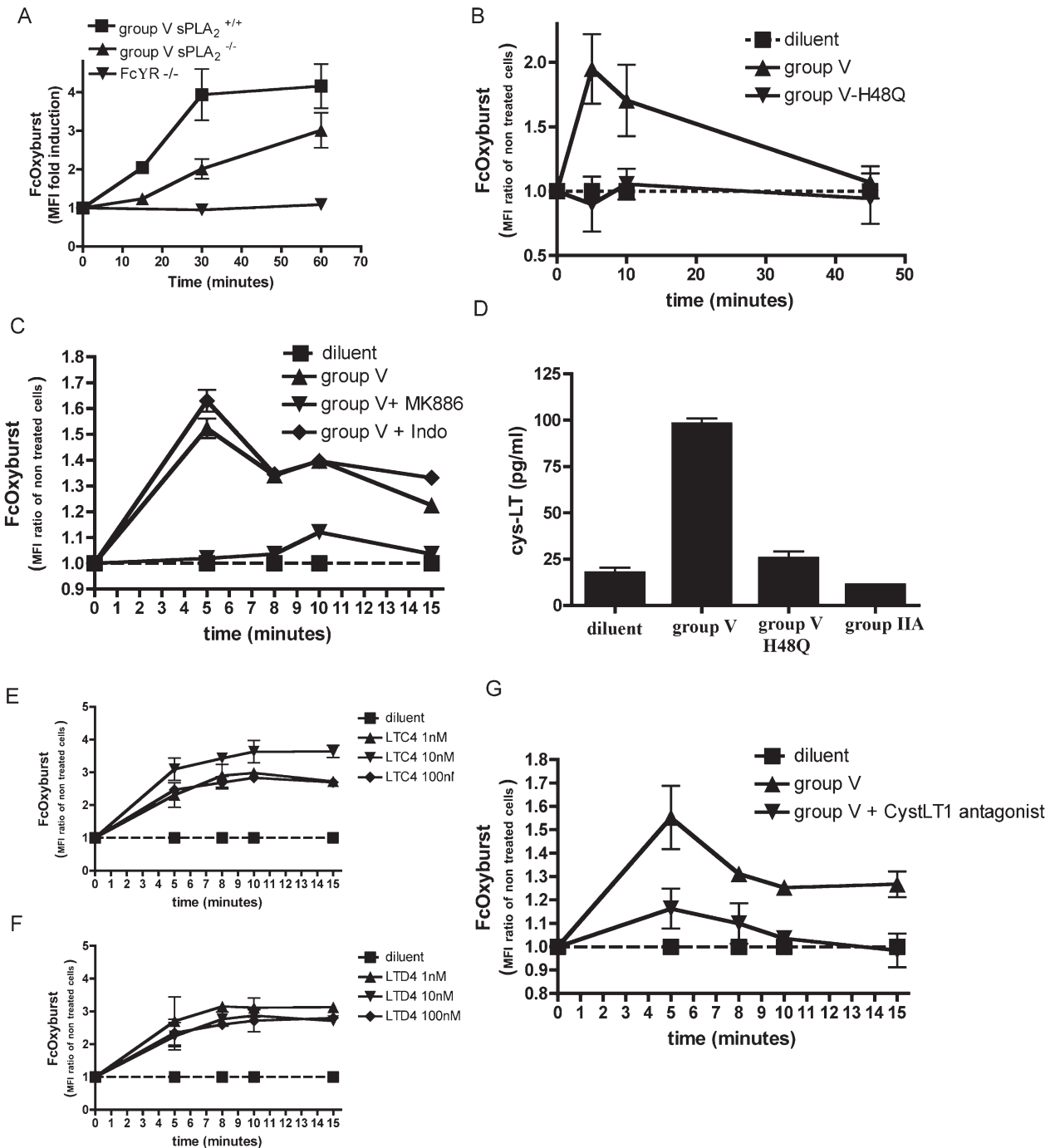
Group V sPLA$_2$ promotes clearance of articular immune complexes *in vivo*

Numerous previous studies demonstrate that IgG immune complexes are found at high levels in joint and SF of patients with RA (Bonomo et al, 1970; Brandt et al, 1968; Britton & Schur,

1971; Fish et al, 1966; Nydegger et al, 1977; Ruddy & Austen, 1970; Ruddy et al, 1975; Schur et al, 1975). Since the K/BxN model also displays articular deposition and pathogenic contributions from immune complexes (Ji et al, 2002a; Matsumoto et al, 2002), we measured IgG and C3 deposition in joint tissues of mice administered K/BxN serum to demonstrate that group V sPLA$_2$ impacts immune complex clearance *in vivo*. Consistent with this mechanistic contribution

by group V sPLA$_2$ to temper the severity of arthritis, we found significantly more articular immune complexes and C3 deposition in mice lacking group V sPLA$_2$ than their congenic WT littermates (Fig 6A and B).

To assess whether group V sPLA$_2$ impacts the systemic metabolism of immune complexes, we quantified circulating immune complexes in WT and group V sPLA$_2$ null mice after administration of K/BxN serum. Interestingly, no differences in

circulating immune complexes were detectable in these mice, pointing to a selective activity for group V sPLA$_2$ in the joint (Fig 6C). The basis for this selectivity did not reside in the phagocytic capacity of tissue resident *versus* circulating phagocytes since we found that group V sPLA$_2$ stimulated comparable phagocytosis of immune complexes by human CD14+ cells, whether from the circulation or from SF (Fig 5 Fig 5B, C and G). Since erythrocytes are absent in SF and abundant in blood, and since the erythrocyte membrane phospholipid composition is well suited for group V sPLA$_2$ binding, we hypothesized that these cells may block group V sPLA$_2$ activity in the circulation. Indeed, addition of small amounts of erythrocytes to the assays wherein group V sPLA$_2$ was administered to phagocytes potently inhibited group V sPLA$_2$ stimulation of cysLT synthesis (Fig 6D) and immune complex phagocytosis (Fig 6E).

DISCUSSION

The anticipated finding in our experiments employing isoform-specific antibodies (Nevalainen et al, 2005), human biospecimens and mice deficient in specific sPLA$_2$ isoforms was an overlapping pro-inflammatory contribution from individual sPLA$_2$ isoforms to inflammatory arthritis. Indeed, group V sPLA$_2$ is potent at releasing arachidonic acid from cell membranes as a substrate for leukotriene synthesis (Kim et al, 2002), and group V sPLA$_2$-deficient cells have impaired eicosanoid synthesis (Kikawada et al, 2007; Satake et al, 2004). Thus, arachidonate release for pro-inflammatory lipid generation was the predominant predicted activity for group V sPLA$_2$ in K/BxN inflammatory arthritis, a model in which eicosanoids contribute (Chen et al, 2006, 2008).

However, our results document a novel counter-regulatory function for group V sPLA$_2$ in inflammatory arthritis. Our mechanistic investigation was guided by our recent observation that group V sPLA$_2$ promotes the phagocytosis of IgG-coated sheep RBC by macrophages (Balestrieri et al, 2009). The demonstration in the current experiments of reduced immune complex phagocytosis in primary macrophages lacking group V sPLA$_2$ and the increase in immune complex deposition in

joint tissues of group V sPLA$_2$ null mice are consistent with an impact of group V sPLA$_2$ on the severity of arthritis by modulating immune complex clearance. This hypothesis was further substantiated by the ability of recombinant group V sPLA$_2$ to stimulate immune complex phagocytosis by CD14+ monocyte/macrophage cells in the circulation and in SF from subjects with RA. Interestingly, the molecular mechanism through which group V sPLA$_2$ regulates the uptake of immune complexes proceeds via its catalytic activity, which promotes cysLT biosynthesis.

While our studies demonstrate a novel anti-inflammatory function for group V sPLA$_2$ in autoimmune inflammatory arthritis, they also point to anatomically or context-dependent actions of this enzyme. On the one hand group V sPLA$_2$ appears to augment early inflammation in acute models of peritonitis (Satake et al, 2004) and in allergic pulmonary inflammation (Munoz et al, 2007). On the other hand, it promotes clearance of pathogens (Balestrieri et al, 2009) and immune complexes. Our observations provide insights that resolve this apparent discrepancy. We find that cysLT generation plays a central role in the promotion of immune complex phagocytosis by group V sPLA$_2$. This is congruent with an earlier study that showed cysLTs stimulated macrophage uptake of IgG-opsonized targets but not of unopsonized particles (Mancuso & Peters-Golden, 2000). Further, we previously documented that synthesis of cysLTs was dispensable to the promotion of macrophage uptake of non-opsonized zymosan yeast particles by group V sPLA$_2$ (Balestrieri et al, 2006). Taken together, an integrated view of these distinct properties is that group V sPLA$_2$ promotes phagocytosis of IgG-opsonized particles or pathogens via generation of cysLTs; it participates in the innate immune response to non-opsonized pathogens via yet to be discovered mechanisms. Moreover, in the pathologic context of chronic immune complex-driven arthritis, where the inflammatory response is inappropriate and sustained, group V sPLA$_2$ can counter-regulate disease activity via its capacity for promoting phagocytic removal of an inciting factor in disease.

Our findings also provide further insight into the activities of tissue macrophages in arthritis. Previous studies examining the role of macrophages in K/BxN serum-transfer arthritis have

Figure 5. Group V sPLA$_2$ promotes clearance of immune complexes *in vitro*.

A. Phagocytosis of immune complexes in vitro by peritoneal macrophages from group V sPLA$_2$-null , group V sPLA$_2$-control, and FcγR-null mice was quantified cytofluorometrically using FcOxyburst. Data are mean ± SEM pooled from three independent experiments.

B. Phagocytosis of immune complexes in vitro by CD14+ cells in RA synovial fluid with or without addition of recombinant group V sPLA$_2$ or its inactive mutant H48Q was quantified cytofluorometrically using FcOxyburst. Data are mean ± SEM pooled from six independent experiments.

C. Phagocytosis of immune complexes by human CD14+ cells from peripheral blood incubated with recombinant group V sPLA$_2$ in the presence of either the cyclooxygenase (COX) inhibitor indomethacin or the FLAP inhibitor MK886. Data are mean ± SEM pooled from three experiments performed in duplicate.

D. CysLTs released by leukocytes from RA SF treated with sPLA$_2$. Group V sPLA$_2$, its mutant H48Q or group IIA sPLA$_2$ were added to leukocytes isolated from RA SF and cysLTs released into the supernatant were quantified by ELISA. Data are mean ± SEM pooled from three experiments performed in duplicate.

E, F. CysLTs promote phagocytosis of immune complexes by CD14+ cells. Indicated concentrations of LTC$_4$ (E) or LTD$_4$ (F) were added to peripheral blood mononuclear cells (PBMC) prior to addition of the FcOxyburst probe and phagocytosis by CD14+ cells was monitored cytofluorometrically. Data are mean ± SEM pooled from three experiments.

G. Phagocytosis of immune complexes by sPLA$_2$-stimulated CD14+ cells from peripheral blood in the presence of cysLT1 antagonist monteleukast. Data are mean ± SEM pooled from three experiments performed in duplicate.
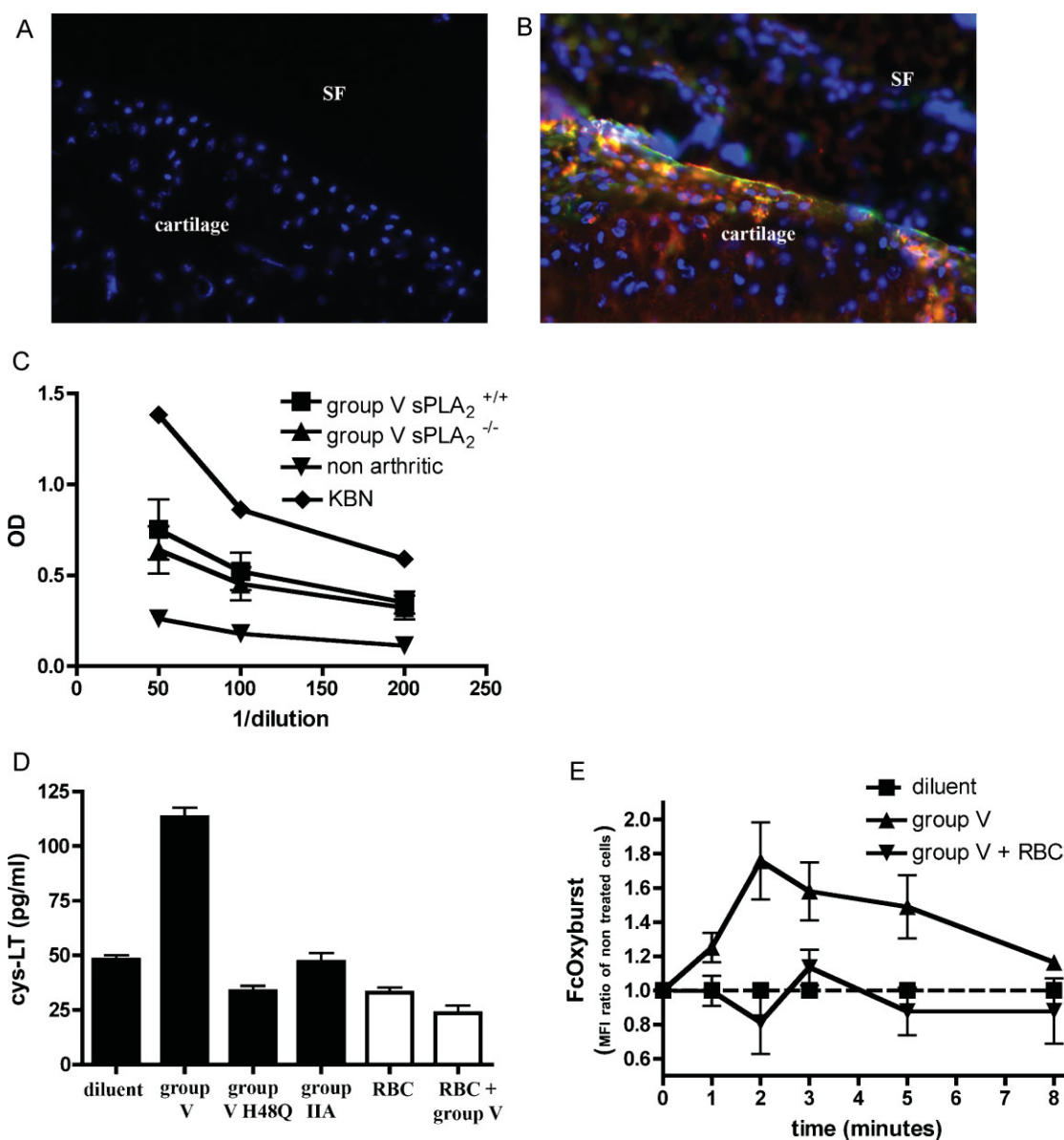
Figure 6. Group V sPLA$_2$ promotes clearance of immune complexes *in vitro*.

A. Immunofluorescent staining of IgG (Red) and complement C3 (Green) in mid-saggital cryosections of ankle tissues from group V sPLA$_2$-control (A) ankle joints. Nuclei (blue) are visualized by staining with DAPI. Magnification= 400X. Cartilage tissue and synovial fluid (SF) space as labeled. Mice were injected with 35 μl K/BxN serum at day 0, and ankle tissues were harvested on day 4. Data are representative of 3 independent experiments.

B. Experiment as detailed in (A) using group V sPLA$_2$-null ankle joints.

C. ELISA quantification of circulating immune complexes in sera from group V sPLA$_2$ null and group V sPLA$_2$ control mice 4 days after administration of 35 μl of K/BxN serum. Pooled K/BxN serum and serum from non-arthritic WT mice were included as controls. $N = 10$ mice/group. Data are mean ± SEM pooled from two independent experiments. $P = $ NS.

D. Quantification of cysLTs released by human PBMC treated with recombinant sPLA$_2$ in the presence (white filled) or absence (black filled) of RBC. Data are mean ± SEM pooled from three experiments.

E. Phagocytosis of immune complexes in the presence of RBC. PBMC incubated in the presence or absence of RBC were treated with group V sPLA$_2$ and phagocytosis of immune complexes by CD14+ cells was monitored cytofluorometrically. Data are mean ± SEM pooled from three experiments.

offered evidence that this lineage contributes to development of K/BxN arthritis (Solomon et al, 2005) and that they also have the capacity to diminish arthritis via activation of the Fc receptor, FcγRIIB (Bruhns et al, 2003). Our findings expand our understanding of murine and human macrophage behaviour in synovitis by documenting a distinct mechanism by which they can impact disease. In addition to their capacity to elaborate soluble mediators of inflammation, it is now apparent that they can alter disease physiology via their prominent phagocyte function.

The contrasting pro-inflammatory and anti-inflammatory properties of group V sPLA$_2$ also raise concern regarding toxicity from exogenously administered enzyme as a potential biotherapeutic. In this context it is notable that adult mice that received exogenous group V sPLA$_2$ did not display overt toxicity and that the pulmonary pathology observed in transgenic mice over-expressing group V sPLA$_2$ (Ohtsuki et al, 2006) was absent in treated mice (Supporting Information Fig S1). We hypothesize that this lack of overt toxicity may be due in part to the capacity for erythrocytes to abrogate the ability of exogenous group V sPLA$_2$ to stimulate cysLT generation. Although not evident in all strains, recent mouse studies also demonstrate an impact of group V sPLA$_2$ on vascular inflammation (Bostrom et al, 2007; Boyanovsky et al, 2009). Thus, extensive assessment of toxicity with chronic parenteral administration of recombinant group V sPLA$_2$ remains warranted prior to investigations in humans.

Group V sPLA$_2$ shares proteoglycan-binding properties with group IIA sPLA$_2$, which is present in increased quantities in the SF of individuals with RA (Fig 1). In contrast to the anti-inflammatory properties of group V sPLA$_2$, our studies in mice deficient in group IIA sPLA$_2$ confirmed the pro-inflammatory actions of group IIA sPLA$_2$ in arthritis. Furthermore, mice transgenic for the human group IIA sPLA$_2$ had an exaggerated inflammatory response and worse clinical disease. Although group IIA sPLA$_2$ has long been implicated as a pro-inflammatory participant in inflammatory arthritis, to the best of our knowledge, these studies are the first to document its functional contribution using a genetic approach. While the pro-inflammatory activity of group IIA sPLA$_2$ awaits clarification, the absence of cysLT release and the lack of phagocytosis stimulation (data not shown) observed with exogenous administration of group IIA sPLA$_2$ to human macrophages points to a distinct mechanism of activity from group V sPLA$_2$. We speculate that differences in the interfacial-binding domains of group V and group IIA sPLA$_2$ may factor prominently in this functional dichotomy. Group IIA sPLA$_2$ prefers membranes rich in anionic phospholipids whereas group V sPLA$_2$ avidly binds phosphatidylcholine (Singer et al, 2002). The external leaflet of mammalian cells is enriched in phosphatidylcholine, thus providing an environment suitable for group V sPLA$_2$. As noted above, further insight into the binding and function of group IIA sPLA$_2$ awaits future investigation.

We also studied mice deficient in group X sPLA$_2$ because this enzyme shares several biochemical properties with group V sPLA$_2$ and has been shown to contribute to arachidonate release and eicosanoid generation (Lambeau & Gelb, 2008). Furthermore, sequence alignment shows ~40% sequence identity

between groups IIA, V and X sPLA$_2$. Structurally, based on the crystal structure for human group IIA and group X sPLA$_2$, all three proteins are thought to share a common interfacial-binding surface and three-dimensional organization (Lambeau & Gelb, 2008; Winget et al, 2006). Despite these similarities in structure and function between the three enzymes, disruption of the gene encoding group X sPLA$_2$ was without effect (either anti- or pro-inflammatory) in the K/BxN serum-transfer model of erosive arthritis (Supporting Information Fig S2). These results underscore the non-redundant function of sPLA$_2$ isoforms and the unique anti-inflammatory participation of group V sPLA$_2$ in autoantibody-driven arthritis.

Finally, these studies provide important new insight into therapeutic targeting of sPLA$_2$ isoforms. The involvement of immune complexes and complement in RA has been extensively documented (reviewed in Nigrovic & Lee, 2006). Therefore, the ability of group V sPLA$_2$ to ameliorate disease severity via stimulation of immune complex phagocytosis by murine and human macrophages suggests its relevance to the pathophysiology of RA and other diseases impacted by immune complexes. Immune complexes are abundantly present in the circulation and tissues of RA patients, while complement activation is evident both by its deposition in RA joint tissues and by depressed levels of intact complement in RA SF (Britton & Schur, 1971; Brodeur et al, 1991; El-Ghobarey & Whaley, 1980; Fostiropoulos et al, 1964; Pekin & Zvaifler, 1964; Ruddy & Austen, 1970; Ruddy et al, 1975; Schur et al, 1975; Zvaifler, 1969). Thus, the newly identified mechanistic and functional activity of group V sPLA$_2$ suggests that its therapeutic administration is a potential novel treatment opportunity for those patients in which immune complexes have a prominent contribution to disease. In addition, our studies demonstrate that the most optimal sPLA$_2$ inhibitor for treatment of RA should be highly selective for group IIA sPLA$_2$. It has been suggested that lack of efficacy of a group IIA sPLA$_2$ inhibitor tested in an RA clinical trial was due to insufficient dosing (Bradley et al, 2005). Our studies raise the additional concern that the inhibitor used in human arthritis trials lacked sufficient specificity (Oslund et al, 2008) and blocked both group IIA sPLA$_2$ and group V sPLA$_2$. Together, our observations provide rationale for pursuing two distinct therapeutic approaches targeted at sPLA$_2$: the use of highly selective group IIA sPLA$_2$ inhibitors and administration of recombinant group V sPLA$_2$.

METHODS

Human synovial fluid analysis

Human knee SF were obtained as discarded material from patients with RA undergoing diagnostic or therapeutic arthrocentesis. RA was diagnosed by an American Board of Internal Medicine certified rheumatologist and/or by review of laboratory, radiologic and clinic notes and by applying ACR classification criteria (Arnett et al, 1988). SF from healthy volunteers was obtained from individuals without prior history of knee trauma, chronic knee pain, prior knee surgery, blood dyscrasias, cancer, chondrocalcinosis, corticosteroid injection or non-steroidal anti-inflammatory drug use in the prior 8 weeks as

described (Gobezie et al, 2007). All studies received Institutional Review Board approval. For time-resolved fluorescence immunoassays of sPLA₂s (Nevalainen et al, 2005), 50 µl of SF were used for all assays except 5 µl was used for group IIA sPLA₂. Assay buffer (50 mM Tris, pH 7.8, 0.9% NaCl, 0.02% Tween-20, 0.05% NaN₃, filtered through a 0.45 µm membrane) was added to each well to bring the total volume to 100 µl. For assay calibration, various amounts of recombinant human sPLA₂ (prepared as described, Singer et al, 2002) were added to assay buffer to generate a standard curve. Blanks were run that contained 100 µl assay buffer alone.

Mice

We used 6–9-week-old mice for all of our studies. All procedures were approved by the Institutional Animal Care and Use Committee of the Dana-Farber Cancer Institute (Boston, MA). Mice were housed in the specific pathogen-free animal facility of the Dana-Farber Cancer Institute. The group IIA sPLA₂ gene in 129 and C57BL/6 mice has a thymidine insertion that disrupts the open reading frame (Kennedy et al, 1995). Group IIA sPLA₂ null mice were produced by backcrossing 129 strain mice (spontaneously group IIA sPLA₂ null) to BALB/cJ mice for 10 generations, selecting offspring heterozygous for disruption of the group IIA sPLA₂ gene. After 10 backcrosses, heterozygous mice were bred to obtain homozygous congenic group IIA sPLA₂ null and WT control mice from which breeding colonies were derived. The 129 allele was detected by polymerase chain reaction amplification of genomic DNA followed by DNA sequencing. Mice lacking group V sPLA₂ were derived from 129 ES cells that lack expression of group IIA sPLA₂, crossed to a BALB/c background for 11 generations as previously described (Satake et al, 2004). Because the genes encoding these enzymes are separated by only ~20 kB, our mice lacking group V sPLA₂ are also deficient in group IIA sPLA₂ (Supporting Information Fig S5 and Table 1). To assess the role of group V sPLA₂ in inflammatory arthritis, we thus backcrossed our sPLA₂-IIA-/V mice onto the BALB/c background and utilized our group IIA sPLA₂ null congenic BALB/c mice as control. The BALB/c and C57BL/6 were obtained from Jackson Laboratory (Bar Harbor, ME). The transgenic human group IIA sPLA₂ mice (Grass et al, 1996) (C57BL/6J background) and the FcRγ null mice (Takai et al, 1994) were obtained from Taconic (Hudson, NY). Group X sPLA₂ null mice on the C57BL6 background were a generous gift from Dr Nancy Webb (in preparation, University of Kentucky, Lexington, KY) and will be described elsewhere.

Recombinant sPLA₂

Recombinant sPLA₂ enzymes were produced as previously described (Rouault et al, 2007; Singer et al, 2002). Proteins were purified to single peaks by high-performance liquid chromatography and both purity as well as appropriate disulphide bond formation was confirmed by SDS–polyacrylamide gel electrophoresis analysis and by electrospray ionization mass spectrometry.

Serum-transfer protocol and arthritis scoring

Arthritogenic K/BxN serum was transferred to recipient mice via intraperitoneal injection on experimental days 0 and 2 to induce arthritis as described (Chen et al, 2008; Korganow et al, 1999). Serum dosing was adjusted based on mouse strain and based on whether the transgenic animals gave an exaggerated or diminished response compared to the WT controls. Ankle thickness was measured at the malleoli with the ankle in a fully flexed position, using spring-loaded dial calipers (Long Island Indicator Service, NY). The clinical index of arthritis was graded on a scale 0–12 as described previously (Chen et al, 2006, 2008; Korganow et al, 1999).

Histological examination

For histomorphometric analysis, ankle tissues were fixed for 24 h in 4% paraformaldehyde in phosphate buffered saline (PBS) and decalcified for 72 h with modified Kristensen's solution. Tissues were then dehydrated, embedded in paraffin, sectioned at 5 µm thickness and stained with haematoxylin and eosin. Histological scoring was performed in a blinded manner as previously described (Chen et al, 2006; Pettit et al, 2001).

Immune complex phagocytosis *in vitro*

Phagocytosis of immune complexes by murine cells

Mouse macrophages were isolated as previously described (Balestrieri et al, 2006), except in Supporting Information Fig S4 in which F4/80+ macrophages were analysed immediately after isolation from the peritoneal cavity. In brief, peritoneal cavities were ushed thrice with 10 ml RPMI containing calcium and magnesium and 10% foetal bovine serum. Cells were then centrifuged and placed in culture in the same medium containing 2 mM ʟ-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in 6-well plates at 37°C, 5% CO₂ for 3 days. Adherent cells were liberated using GIBCO™ cell dissociation buffer enzyme free (Invitrogen) and resuspended in Kreb's Ringer phosphate (KRP) buffer (PBS containing 1 mM CaCl₂, 1.5 mM MgCl₂, 5.5 mM glucose, pH 7.4). Phagocytosis was quantied using BSA-anti-BSA immune complexes in which bovine serum albumin (BSA) is covalently bound to dichlorodihydrouorescein (FcOxyburst, Invitrogen). In this method, FcγR-mediated internalization of immune complexes leads to an oxidative burst in the phagosomal vacuole that is monitored cytouorometrically as described in the manufacturer's protocol and is exemplied in Supporting Information Fig S6. Cells (2×10^6/ml) were incubated at 37°C with the FcOxyburst probe (60 µg/ml), and development of uorescence was monitored at the indicated time points. As a negative control, macrophages from FcγR$^{-/-}$ mice were analysed for phagocytosis. Data are presented as a ratio between the mean uorescence intensity (MFI) at the indicated time and the MFI at the beginning of the reaction ($t = 0$). For each time point, a minimum of 5000 macrophages was analysed, in duplicate, in three independent experiments.

Table 1. Mouse strain expression of sPLA₂ isoforms

Mouse strain	Group IIA sPLA₂	Group V sPLA₂
BALB/c	+/+	+/+
C57BL/6	−/−	+/+
129	−/−	+/+
Group V sPLA₂ null (BALB/c background)	−/−	−/−
Group V sPLA₂ congenic control (BALB/c background)	−/−	+/+

The paper explained

PROBLEM:

Prior studies have implicated secretory phospholipase A$_2$ (sPLA$_2$) in the inflammatory response in rheumatoid arthritis (RA). However, these studies were performed before the size and diversity of this family of enzymes was appreciated and therefore the exact sPLA$_2$ isoforms present in RA synovial fluid (SF) and their *in vivo* roles in the disease remain unclear.

RESULTS:

Several sPLA$_2$ species are present in the SF in RA. Importantly, we demonstrate an unexpected and novel anti-inflammatory function for group V sPLA$_2$ in autoimmune arthritis.

sPLA$_2$-V promotes the phagocytic uptake of immune complexes by macrophages *in vitro* and reduces the deposition of articular immune complexes with amelioration of the inflammatory response *in vivo*.

IMPACT:

The data highlight the diversity of roles of sPLA$_2$ in autoimmunity and inflammation and underscore the importance of developing selective sPLA$_2$ inhibitors in RA therapy. In addition, the study pinpoints group V sPLA$_2$ as a novel potential biotherapeutic for the treatment of arthritis.

Phagocytosis of immune complexes by human cells

Human PBMC were isolated by centrifugation of ethylenediaminetetraacetic acid anticoagulated human blood obtained from healthy donors on Ficoll-Paque Premiun (GE Healthcare) as described by the manufacturer. PBMC were washed in PBS and resuspended in KRP buffer (4×10^6/ml). For phagocytosis by human cells contained in freshly collected RA SF, cells were washed in PBS and the cell concentration was adjusted to 4×10^6/ml in KRP buffer. Typically, RA SF comprised $\sim 10 \times 10^6$/ml leukocytes, $22.9 \pm 4\%$ being CD14+. 5-Lipoxygenase activating protein (FLAP) inhibitor MK886 (5 μM) (Cayman), COX inhibitor indomethacin (1 μM) (Sigma), CysLT1 antagonist monteleukast (5 μM) (Cayman) were added 5 min prior to sPLA$_2$ addition. Recombinant human group V sPLA$_2$, its inactive mutant H48Q group V sPLA$_2$ and group IIA sPLA$_2$ (5 μg/ml) were added 20 min before addition of the FcOxyburst reagent (60 μg/ml). At the indicated time points, cells were transferred to tubes containing 300 μl cold PBS and phycoerythrin (PE)-labelled anti-CD14 (BD Pharmingen) and kept on ice until the cytouorometric analysis were performed. The monocyte/macrophage population was dened by CD14 staining. A minimum of 5000 cells were analysed in each of six independent experiments.

Phagocytosis in the presence of RBC

Efforts were made to mimic the abundance of RBC in blood and to successfully limit the interference and excess of RBC could have on binding of FcOxyburst probe to macrophages Fc receptors. Autologous RBC washed in PBS (500 RBC:1 CD14+ cells) were added to puried PBMC and incubated with group V sPLA$_2$ for 20 min at 37°C in KRP buffer. Cells were next transferred to 4°C for 10 min and incubated further for 25 min with the FcOxyburst probe (60 μg/ml) to allow saturation of macrophages Fc receptors. Cells were next transferred to 37°C and the reaction started. At the indicated time points, cells were transferred to tubes containing 300 μl cold PBS and PE-labelled anti-CD14 and kept on ice until the cytouorometric analysis were performed. The monocyte/macrophage population was dened by

CD14 staining. A minimum of 5000 cells was analysed in each of three independent experiments.

Detection of immune complexes *in vivo*

Joint immune complexes

Snap frozen ankles were prepared from mice 4 days after injection with K/BxN serum. Cryostat sections from non-xed, non-decalcied ankle joints were generated using a tape capture technique as described (Ji et al, 2002a; Watts et al, 2005). After blocking with 2% BSA and 0.04% Tween in PBS, the sections were incubated with texas-red-conjugated anti-mouse IgG (Jackson Immunoresearch), and FITC-conjugated goat anti-mouse C3 (ICN Biomedicals, Costa Mesa, CA) or control IgG (500 ng/section). Fluorescence was detected by microscopy (Nikon Eclipse E800). Yellow staining denes colocalization of C3 and IgG. Nuclei (blue) were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (50 ng/section, Molecular Probes). Images were acquired (Camera from Diagnostic Instruments) and processed digitally (Photoshop 6.0).

Circulating immune complexes

Immune complexes in serum were detected by enzyme-linked immunosorbent assay (ELISA) (Matsumoto et al, 2002). In this method, target C1q (20 μg/ml) (Sigma) in PBS was added to 96-well ELISA plates (Nunc) for 18 h at 4°C. The wells were blocked with 1% BSA in PBS, after which sera obtained from mice 4 days after the administration of K/BxN serum and diluted as indicated were added. Pooled serum from K/BxN mice was used as a positive control while sera from mice not administered K/BxN serum were used as a negative control. Bound complexes were detected using an horseradish peroxidase-coupled anti-mouse IgG (Jackson Immunoresearch).

Measurements of cysteinyl leukotrienes synthesis

CysLTs were measured in supernatants of cells treated with group V sPLA$_2$, its inactive mutant group V sPLA$_2$-H48Q and group IIA sPLA$_2$ (5 μg/ml) in KRP buffer for 30 min at 37°C using a commercial ELISA (Cayman) according to the manufacturer's instructions.

Statistical analysis

Mouse arthritis experiments are presented as mean $\pm$ SEM. The statistical significance for comparisons between groups was determined using two-way analysis of variance, followed by Bonferroni correction using Prism software package 4.00 (GraphPAd Software, San Diego, CA). Comparison of $sPLA_2$ content between RA and normal SF was made by Student's *t*-test. *P*-values smaller than 0.05 were considered significant. Spearman rho was calculated to assess correlation between group-IIA and group-V $sPLA_2$ in SF where expression of both isoforms was detected using the Prism software package.

Author contributions

Eric Boilard: Study conception, study design, acquisition of data, analyses and interpretation of data, manuscript preparation, statistical analyses.

Ying Lai: Acquisition of data, analyses and interpretation of data.

Katherine Larabee: Acquisition of data, analyses and interpretation of data, manuscript preparation.

Barbara Balestrieri: Generated critical reagent (group IIA $sPLA_2$ null mice on a BALB/c background), manuscript preparation.

Farideh Ghomashchi: Acquisition of data, analyses and interpretation of data.

Daisuke Fujioka: Contributed important reagent, manuscript preparation.

Reuben Gobezie: Acquisition of data, manuscript preparation.

Jonathan S. Coblyn: Acquisition of data, manuscript preparation.

Michael E. Weinblatt: Acquisition of data, manuscript preparation.

Elena M. Massarotti: Acquisition of data, manuscript preparation.

Thomas S. Thornhill: Acquisition of data, manuscript preparation.

Maziar Divangahi: Acquisition of data, manuscript preparation.

Heinz Remold: Analyses and interpretation of data, manuscript preparation.

Gérard Lambeau: Analyses and interpretation of data, manuscript preparation.

Michael H. Gelb: Study design, acquisition of data, analyses and interpretation of data, manuscript preparation.

Jonathan P. Arm: Study design, analyses and interpretation of data, manuscript preparation.

David M. Lee: Study conception, study design, acquisition of data, analyses and interpretation of data, manuscript preparation.

Acknowledgements

Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

References

Arnesjo B, Barrowman J, Borgstrom B (1967) The zymogen of phospholipase A2 in rat pancreatic juice. Acta Chem Scand 21: 2897-2900

Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, *et al* (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31: 315-324

Balestrieri B, Hsu VW, Gilbert H, Leslie CC, Han WK, Bonventre JV, Arm JP (2006) Group V secretory phospholipase A2 translocates to the phagosome after zymosan stimulation of mouse peritoneal macrophages and regulates phagocytosis. J Biol Chem 281: 6691-6698

Balestrieri B, Maekawa A, Xing W, Gelb MH, Katz HR, Arm JP (2009) Group V secretory phospholipase A2 modulates phagosome maturation and regulates the innate immune response against *Candida albicans*. J Immunol 182: 4891-4898

Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, Massarotti EM, Remold-O'Donnell E, Farndale RW, Ware J, *et al* (2010) Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. Science 327: 580-583

Bonomo L, Tursi A, Trizio D, Gillardi U, Dammacco F (1970) Immune complexes in rheumatoid synovitis: a mixed staining immunofluorescence study. Immunology 18: 557-563

Bonventre JV, Huang Z, Taheri MR, O'Leary E, Li E, Moskowitz MA, Sapirstein A (1997) Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2. Nature 390: 622-625

Bostrom MA, Boyanovsky BB, Jordan CT, Wadsworth MP, Taatjes DJ, de Beer FC, Webb NR (2007) Group V secretory phospholipase A2 promotes atherosclerosis: evidence from genetically altered mice. Arterioscler Thromb Vasc Biol 27: 600-606

Boyanovsky B, Zack M, Forrest K, Webb NR (2009) The capacity of group V sPLA2 to increase atherogenicity of ApoE$-/-$ and LDLR$-/-$ mouse LDL in vitro predicts its atherogenic role in vivo. Arterioscler Thromb Vasc Biol 29: 532-538

Bradley JD, Dmitrienko AA, Kivitz AJ, Gluck OS, Weaver AL, Wiesenhutter C, Myers SL, Sides GD (2005) A randomized, double-blinded, placebo-controlled clinical trial of LY333013, a selective inhibitor of group II secretory phospholipase A2, in the treatment of rheumatoid arthritis. J Rheumatol 32: 417-423

Brandt KD, Cathcart ES, Cohen AS (1968) Studies of immune deposits in synovial membranes and corresponding synovial fluids. J Lab Clin Med 72: 631-647

Britton MC, Schur PH (1971) The complement system in rheumatoid synovitis. II. Intracytoplasmic inclusions of immunoglobulins and complement. Arthritis Rheum 14: 87-95

Brodeur JP, Ruddy S, Schwartz LB, Moxley G (1991) Synovial fluid levels of complement SC5b-9 and fragment Bb are elevated in patients with rheumatoid arthritis. Arthritis Rheum 34: 1531-1537

Bruhns P, Samuelsson A, Pollard JW, Ravetch JV (2003) Colony-stimulating factor-1-dependent macrophages are responsible for IVIG protection in antibody-induced autoimmune disease. Immunity 18: 573-581

Chen M, Lam BK, Kanaoka Y, Nigrovic PA, Audoly LP, Austen KF, Lee DM (2006) Neutrophil-derived leukotriene B4 is required for inflammatory arthritis. J Exp Med 203: 837-842

Chen M, Boilard E, Nigrovic PA, Clark P, Xu D, Fitzgerald GA, Audoly LP, Lee DM (2008) Predominance of cyclooxygenase 1 over cyclooxygenase 2 in the generation of proinflammatory prostaglandins in autoantibody-driven K/BxN serum-transfer arthritis. Arthritis Rheum 58: 1354-1365

Chiba A, Kaieda S, Oki S, Yamamura T, Miyake S (2005) The involvement of V(alpha)14 natural killer T cells in the pathogenesis of arthritis in murine models. Arthritis Rheum 52: 1941-1948

Corr M, Crain B (2002) The role of FcgammaR signaling in the K/BxN serum transfer model of arthritis. J Immunol 169: 6604-6609

El-Ghobarey A, Whaley K (1980) Alternative pathway complement activation in rheumatoid arthritis. J Rheumatol 7: 453-460

Fish AJ, Michael AF, Gewurz H, Good RA (1966) Immunopathologic changes in rheumatoid arthritis synovium. Arthritis Rheum 9: 267-280

Foreman-Wykert AK, Weinrauch Y, Elsbach P, Weiss J (1999) Cell-wall determinants of the bactericidal action of group IIA phospholipase A2 against Gram-positive bacteria. J Clin Invest 103: 715-721

Fostiropoulos G, Austen KF, Bloch KJ (1964) Total hemolytic complement and second component of complement activity in serum and synovial fluid of patients with rheumatic diseases. Arthritis Rheum 7: 308

Funakoshi A, Yamada Y, Migita Y, Wakasugi H (1993) Simultaneous determinations of pancreatic phospholipase A2 and prophospholipase A2 in various pancreatic diseases. Dig Dis Sci 38: 502-506

Gobezie R, Kho A, Krastins B, Sarracino DA, Thornhill TS, Chase M, Millett PJ, Lee DM (2007) High abundance synovial fluid proteome: distinct profiles in health and osteoarthritis. Arthritis Res Ther 9: R36

Grass DS, Felkner RH, Chiang MY, Wallace RE, Nevalainen TJ, Bennett CF, Swanson ME (1996) Expression of human group II PLA2 in transgenic mice results in epidermal hyperplasia in the absence of inflammatory infiltrate. J Clin Invest 97: 2233-2241

Green JA, Smith GM, Buchta R, Lee R, Ho KY, Rajkovic IA, Scott KF (1991) Circulating phospholipase A2 activity associated with sepsis and septic shock is indistinguishable from that associated with rheumatoid arthritis. Inflammation 15: 355-367

Haworth O, Cernadas M, Yang R, Serhan CN, Levy BD (2008) Resolvin E1 regulates interleukin 23, interferon-gamma and lipoxin A4 to promote the resolution of allergic airway inflammation. Nat Immunol 9: 873-879

Hegen M, Sun L, Uozumi N, Kume K, Goad ME, Nickerson-Nutter CL, Shimizu T, Clark JD (2003) Cytosolic phospholipase A2alpha-deficient mice are resistant to collagen-induced arthritis. J Exp Med 197: 1297-1302

Hegen MLJ, Shen M, Stedman N, Leach MW, Collins M, Shimizu T, Clark JD, Nicker-Nutter CL (2006) Cytosolic Phospholipase A2α-Deficient Mice are Resistant to Arthritis in the K/BxN Serum Transfer Model. *The 70th Annual Scientific Meeting of the American College of Rheumatology*, Atlanta, GA, Presentation number: 314

Huggins KW, Boileau AC, Hui DY (2002) Protection against diet-induced obesity and obesity-related insulin resistance in Group 1B PLA2-deficient mice. Am J Physiol Endocrinol Metab 283: E994-E1001

Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, Boackle SA, Takahashi K, Holers VM, Walport M, Gerard C, et al (2002a) Arthritis critically dependent on innate immune system players. Immunity 16: 157-168

Ji H, Pettit A, Ohmura K, Ortiz-Lopez A, Duchatelle V, Degott C, Gravallese E, Mathis D, Benoist C (2002b) Critical roles for interleukin 1 and tumor necrosis factor alpha in antibody-induced arthritis. J Exp Med 196: 77-85

Kennedy BP, Payette P, Mudgett J, Vadas P, Pruzanski W, Kwan M, Tang C, Rancourt DE, Cromlish WA (1995) A natural disruption of the secretory group II phospholipase A2 gene in inbred mouse strains. J Biol Chem 270: 22378-22385

Kikawada E, Bonventre JV, Arm JP (2007) Group V secretory PLA2 regulates TLR2-dependent eicosanoid generation in mouse mast cells through amplification of ERK and cPLA2alpha activation. Blood 110: 561-567

Kim YJ, Kim KP, Han SK, Munoz NM, Zhu X, Sano H, Leff AR, Cho W (2002) Group V phospholipase A2 induces leukotriene biosynthesis in human neutrophils through the activation of group IVA phospholipase A2. J Biol Chem 277: 36479-36488

Kim ND, Chou RC, Seung E, Tager AM, Luster AD (2006) A unique requirement for the leukotriene B4 receptor BLT1 for neutrophil recruitment in inflammatory arthritis. J Exp Med 203: 829-835

Kini RM (2003) Excitement ahead: structure, function and mechanism of snake venom phospholipase A2 enzymes. Toxicon 42: 827-840

Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, Degott C, Kikutani H, Rajewsky K, Pasquali JL, et al (1999) From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. Immunity 10: 451-461

Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D (1996) Organ-specific disease provoked by systemic autoimmunity. Cell 87: 811-822

Kudo I, Murakami M (2002) Phospholipase A2 enzymes. Prostaglandins Other Lipid Mediat 68–69: 3-58

Lambeau G, Gelb MH (2008) Biochemistry and physiology of mammalian secreted phospholipases A(2). Annu Rev Biochem 77: 495-520

Lee DM, Friend DS, Gurish MF, Benoist C, Mathis D, Brenner MB (2002) Mast cells: a cellular link between autoantibodies and inflammatory arthritis. Science 297: 1689-1692

Mancuso P, Peters-Golden M (2000) Modulation of alveolar macrophage phagocytosis by leukotrienes is Fc receptor-mediated and protein kinase C-dependent. Am J Respir Cell Mol Biol 23: 727-733

Matsumoto I, Staub A, Benoist C, Mathis D (1999) Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. Science 286: 1732-1735

Matsumoto I, Maccioni M, Lee DM, Maurice M, Simmons B, Brenner M, Mathis D, Benoist C (2002) How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease. Nat Immunol 3: 360-365

Menschikowski M, Hagelgans A, Siegert G (2006) Secretory phospholipase A2 of group IIA: Is it an offensive or a defensive player during atherosclerosis and other inflammatory diseases? Prostaglandins Other Lipid Mediat 79: 1-33

Mounier CM, Wendum D, Greenspan E, Flejou JF, Rosenberg DW, Lambeau G (2008) Distinct expression pattern of the full set of secreted phospholipases A2 in human colorectal adenocarcinomas: sPLA2-III as a biomarker candidate. Br J Cancer 98: 587-595

Munoz NM, Meliton AY, Arm JP, Bonventre JV, Cho W, Leff AR (2007) Deletion of secretory group V phospholipase A2 attenuates cell migration and airway hyperresponsiveness in immunosensitized mice. J Immunol 179: 4800-4807

Munoz NM, Meliton AY, Meliton LN, Dudek SM, Leff AR (2009) Secretory group V phospholipase A2 regulates acute lung injury and neutrophilic inflammation caused by LPS in mice. Am J Physiol Lung Cell Mol Physiol 296: L879-887

Nagiec MJ, Lei B, Parker SK, Vasil ML, Matsumoto M, Ireland RM, Beres SB, Hoe NP, Musser JM (2004) Analysis of a novel prophage-encoded group A *Streptococcus* extracellular phospholipase A(2). J Biol Chem 279: 45909-45918

Nevalainen TJ, Eerola LI, Rintala E, Laine VJ, Lambeau G, Gelb MH (2005) Time-resolved fluoroimmunoassays of the complete set of secreted phospholipases A2 in human serum. Biochim Biophys Acta 1733: 210-223

Nigrovic PA, Lee DM (2006) Immune complexes and innate immunity in rheumatoid arthritis. In: *Rheumatoid Arthritis: New Frontiers in Pathogenesis and Treatment*, 2nd edn, Firestein GS, Panayi GS, and Wollheim FA (eds), Oxford, Oxford University Press: pp 135-156

Nydegger UE, Zubler RH, Gabay R, Joliat G, Karagevrekis CH, Lambert PH, Miescher PA (1977) Circulating complement breakdown products in patients with rheumatoid arthritis. Correlation between plasma C3d, circulating immune complexes, and clinical activity. J Clin Invest 59: 862-868

Ohtsuki M, Taketomi Y, Arata S, Masuda S, Ishikawa Y, Ishii T, Takanezawa Y, Aoki J, Arai H, Yamamoto K, et al (2006) Transgenic expression of group V,

but not group X, secreted phospholipase A2 in mice leads to neonatal lethality because of lung dysfunction. J Biol Chem 281: 36420-36433

Oslund RC, Cermak N, Gelb MH (2008) Highly specific and broadly potent inhibitors of mammalian secreted phospholipases A2. J Med Chem 51: 4708-4714

Pekin TJ, Jr, Zvaifler NJ (1964) Hemolytic complement in synovial fluid. J Clin Invest 43: 1372-1382

Pettit AR, Ji H, von Stechow D, Muller R, Goldring SR, Choi Y, Benoist C, Gravallese EM (2001) TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. Am J Pathol 159: 1689-1699

Piris-Gimenez A, Paya M, Lambeau G, Chignard M, Mock M, Touqui L, Goossens PL (2005) In vivo protective role of human group IIa phospholipase A2 against experimental anthrax. J Immunol 175: 6786-6791

Pruzanski W, Vadas P, Stefanski E, Urowitz MB (1985) Phospholipase A2 activity in sera and synovial fluids in rheumatoid arthritis and osteoarthritis. Its possible role as a proinflammatory enzyme. J Rheumatol 12: 211-216

Pruzanski W, Scott K, Smith G, Rajkovic I, Stefanski E, Vadas P (1992) Enzymatic activity and immunoreactivity of extracellular phospholipase A2 in inflammatory synovial fluids. Inflammation 16: 451-457

Rouault M, Le Calvez C, Boilard E, Surrel F, Singer A, Ghomashchi F, Bezzine S, Scarzello S, Bollinger J, Gelb MH, *et al* (2007) Recombinant production and properties of binding of the full set of mouse secreted phospholipases A2 to the mouse M-type receptor. Biochemistry 46: 1647-1662

Ruddy S, Austen KF (1970) The complement system in rheumatoid synovitis. I. An analysis of complement component activities in rheumatoid synovial fluids. Arthritis Rheum 13: 713-723

Ruddy S, Fearon DT, Austen KF (1975) Depressed synovial fluid levels of properdin and properdin factor B in patients with rheumatoid arthritis. Arthritis Rheum 18: 289-295

Satake Y, Diaz BL, Balestrieri B, Lam BK, Kanaoka Y, Grusby MJ, Arm JP (2004) Role of group V phospholipase A2 in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption. J Biol Chem 279: 16488-16494

Schaloske RH, Dennis EA (2006) The phospholipase A2 superfamily and its group numbering system. Biochim Biophys Acta 1761: 1246-1259

Schur PH, Britton MC, Franco AE, Corson JM, Sosman JL, Ruddy S (1975) Rheumatoid synovitis: complement and immune complexes. Rheumatology 6: 34-42

Schwab JM, Serhan CN (2006) Lipoxins and new lipid mediators in the resolution of inflammation. Curr Opin Pharmacol 6: 414-420

Schwab JM, Chiang N, Arita M, Serhan CN (2007) Resolvin E1 and protectin D1 activate inflammation-resolution programmes. Nature 447: 869-874

Seilhamer JJ, Plant S, Pruzanski W, Schilling J, Stefanski E, Vadas P, Johnson LK (1989a) Multiple forms of phospholipase A2 in arthritic synovial fluid. J Biochem 106: 38-42

Seilhamer JJ, Pruzanski W, Vadas P, Plant S, Miller JA, Kloss J, Johnson LK (1989b) Cloning and recombinant expression of phospholipase A2 present in rheumatoid arthritic synovial fluid. J Biol Chem 264: 5335-5338

Shin K, Nigrovic PA, Crish J, Boilard E, McNeil HP, Larabee KS, Adachi R, Gurish MF, Gobezie R, Stevens RL, *et al* (2009) Mast cells contribute to autoimmune inflammatory arthritis via their tryptase/heparin complexes. J Immunol 182: 647-656

Singer AG, Ghomashchi F, Le Calvez C, Bollinger J, Bezzine S, Rouault M, Sadilek M, Nguyen E, Lazdunski M, Lambeau G, *et al* (2002) Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A2. J Biol Chem 277: 48535-48549

Solomon S, Rajasekaran N, Jeisy-Walder E, Snapper SB, Illges H (2005) A crucial role for macrophages in the pathology of K/BxN serum-induced arthritis. Eur J Immunol 35: 3064-3073

Soragni E, Bolchi A, Balestrini R, Gambaretto C, Percudani R, Bonfante P, Ottonello S (2001) A nutrient-regulated, dual localization phospholipase A(2) in the symbiotic fungus *Tuber borchii*. EMBO J 20: 5079-5090

Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV (1994) FcR gamma chain deletion results in pleiotrophic effector cell defects. Cell 76: 519-529

Uozumi N, Kume K, Nagase T, Nakatani N, Ishii S, Tashiro F, Komagata Y, Maki K, Ikuta K, Ouchi Y, *et al* (1997) Role of cytosolic phospholipase A2 in allergic response and parturition. Nature 390: 618-622

Vadas P, Stefanski E, Pruzanski W (1985) Characterization of extracellular phospholipase A2 in rheumatoid synovial fluid. Life Sci 36: 579-587

Valentin E, Lambeau G (2000) Increasing molecular diversity of secreted phospholipases A(2) and their receptors and binding proteins. Biochim Biophys Acta 1488: 59-70

Watts GM, Beurskens FJ, Martin-Padura I, Ballantyne CM, Klickstein LB, Brenner MB, Lee DM (2005) Manifestations of inflammatory arthritis are critically dependent on LFA-1. J Immunol 174: 3668-3675

Winget JM, Pan YH, Bahnson BJ (2006) The interfacial binding surface of phospholipase A2s. Biochim Biophys Acta 1761: 1260-1269

Wipke BT, Allen PM (2001) Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. J Immunol 167: 1601-1608

Zadori Z, Szelei J, Lacoste MC, Li Y, Gariepy S, Raymond P, Allaire M, Nabi IR, Tijssen P (2001) A viral phospholipase A2 is required for parvovirus infectivity. Dev Cell 1: 291-302

Zvaifler NJ (1969) Breakdown products of C3 in human synovial fluids. J Clin Invest 48: 1532-1542