A novel anti-inflammatory role for secretory phospholipase A2 in immune complex-mediated arthritis

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Phospholipase A2 (PLA2) 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 PLA2 enzymes (sPLA2), 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 sPLA2 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 sPLA2 isoform plays a novel anti-inflammatory role that opposes the pro-inflammatory activity of group IIA sPLA2. Mechanistically, group V sPLA2 counter-regulation includes promotion of immune complex clearance by regulating cysteinyl leukotriene synthesis. These observations identify a novel anti-inflammatory function for a PLA2 and identify group V sPLA2 as a potential biotherapeutic for treatment of immune-complex-mediated inflammation.

INTRODUCTION

Phospholipase A2 (PLA2) 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 PLA2 isoforms that have been grouped into three major classes, namely the calcium-dependent and -independent intracellular enzymes, and calcium-dependent secreted PLA2 (sPLA2) (Schalses & Dennis, 2006; Valentin & Lambeau, 2000). The best characterized member of the PLA2 family is cytosolic group IV PLA2α, which is constitutively expressed in most tissues. Deletion of group IV cPLA2α 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 sPLA2 family members are strikingly diverse. They are typically ~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 sPLA2s (nine of which are expressed in humans) and two sPLA2-like proteins devoid of catalytic activity.

sPLA2 enzymes have been implicated in physiological functions, host defence and inflammation. Group IB sPLA2 is present in pancreatic secretions and exhibits a role in dietary phospholipid digestion (Arnesjo et al, 1967; Huggins et al, 2002). Group IIA sPLA2 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 sPLA2 also plays a role in host defence by promoting phagocytosis and killing of fungal species (Balestrieri et al, 2006, 2009).

sPLA2 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 sPLA2 in these processes. However, many of these studies were performed prior to the characterization of the multiple sPLA2 isoforms and before the development of isoform-specific reagents. Recent descriptions of the pro-inflammatory role of group V sPLA2 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 sPLA2 isoforms in RA synovial fluid (SF) and to evaluate the role of group V sPLA2 in arthritis. Surprisingly, our data indicate a counter-regulatory role for group V sPLA2 in a mouse model of immune complex-mediated arthritis. Further, we delineate a novel mechanism, shared in mice and humans, in which group V sPLA2 promotes the phagocytosis of immune complexes by macrophages to ameliorate inflammation. This group V sPLA2 function depends on its enzymatic activity and the generation of cysteinyi leukotrienes (cysLTs). Interestingly, we also confirm the pro-inflammatory role of group IIA sPLA2 in arthritis.

These findings highlight the complexity of sPLA2 species’ participation in inflammation, reveal a previously unappreciated and unanticipated anti-inflammatory function for group V sPLA2, underscore the importance of developing selective inhibitors of pro-inflammatory sPLA2 members for use in therapy and identify group V sPLA2 as a novel potential biotherapeutic in arthritis.

RESULTS

Diverse sPLA2 are detected in RA synovial fluids

Given recent insights into distinct functional activities for sPLA2 species, and since most analyses of PLA2 enzymatic activity in SF were performed prior to cloning the complete family of sPLA2 isoforms (Pruzanski et al, 1992, 1985; Seilhamer et al, 1989a; Vadas et al, 1985), we quantified the concentration of all catalytically active human sPLA2 in SF from 45 subjects with RA using a specific immunoassay that distinguishes the different sPLA2 isoforms (Fig 1A).
Figure 2. Group V sPLA₂ protects from K/BxN serum-transfer arthritis.

A. Arthritis response in group V sPLA₂ null and congenic control mice in a BALB/c group II A 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 sPLA₂ null and congenic control mice at experimental day 13. N = 15 mice/group. Data are mean ± SEM pooled from three independent experiments. *P < 0.001 for (A).

C. Representative mid-saggital ankle sections from group V sPLA₂ null and group V sPLA₂ control mice. Upper and lower panels are 25× and 200× 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 sPLA₂ null mice (T, tibia; S, synovial space; C, calcaneus). Figures representative of findings in 15 mice/group.
Interestingly, all sPLA₂ isoforms could be detected, although typically each isoform was expressed in only a subgroup of subjects.

To examine disease-associated differences in sPLA₂ isoform expression, we also quantified sPLA₂ 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 sPLA₂. Group IIA sPLA₂ was more prominently expressed than group V sPLA₂ 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 sPLA₂ demonstrates no statistically significant correlation between isoform levels in RA SF ($r = 0.3497$, $P = 0.1326$),
sugest that the expression of the two enzymes is independently regulated.

**Anti-inflammatory activity of group V sPLA2 in autoimmune arthritis**

Given the failed clinical trial of a putative group IIA sPLA2 inhibitor in arthritis (Bradley et al., 2005) and having demonstrated expression of group V sPLA2 in arthritic human SF, we examined the contribution of group V sPLA2 to inflammatory arthritis. We employed the K/BxN serum-transfer model of autoimmune inflammatory arthritis to explore the contributions of group V sPLA2 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 Aβ7 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 (LTB4 and PGI2) 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 sPLA2 in inflammatory arthritis, we examined the response of group V sPLA2 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 sPLA2 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 sPLA2 null mice (Fig 2B and C).

**Systemic administration of recombinant group V sPLA2 ameliorates arthritis**

To validate the modulating role of endogenous group V sPLA2 in antibody-driven inflammatory arthritis and to provide in vivo proof of concept for therapeutic use of group V sPLA2, we produced highly purified recombinant mouse group V sPLA2 and administered this material parenterally to group V sPLA2 null mice. Mice deficient in group V sPLA2 treated with recombinant group V sPLA2 were substantially protected from K/BxN arthritis (Fig 3A, C and D). Interestingly, parenteral administration of recombinant group V sPLA2 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 sPLA2 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 sPLA2, previous studies in group V sPLA2 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 sPLA2, and found no evidence for tissue abnormality (Supporting Information Fig S1).

**Group IIA sPLA2 contributes to synovial inflammation**

Since the anti-inflammatory activity of group V sPLA2 was unexpected, we expanded our analyses by investigating whether group IIA sPLA2 displayed an expected pro-inflammatory role in disease. As anticipated, mice with an isolated deficiency in group IIA sPLA2 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 sPLA2 null mice (Fig 4D).

Although most closely related by sequence homology (Selhamer et al., 1989b), whether human group IIA sPLA2 is the functional orthologue of murine group IIA sPLA2 remains
Figure 4. Group IIA sPLA2 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 sPLA2 null and WT congenic BALB/c control mice.

B. Histomorphometric quantification of arthritis severity in group IIA sPLA2 null and congenic BALB/c control mice at experimental day 13. N = 15 mice/group. Data are mean ± SEM pooled from three independent experiments. P < 0.001 (A).

C. Human group IIA sPLA2 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 sPLA2 transgenic and WT control mice at experimental day 13. N = 15 mice/group. Data are mean ± SEM pooled from three independent experiments. P < 0.001 for (C).
speculative. To confirm the pro-inflammatory contribution of human group IIA sPLA2 to synovitis, we assessed the severity of K/BxN arthritis in mice expressing a human group IIA sPLA2 transgene (Grass et al, 1996). Because the C57BL/6 (B6) strain contains a spontaneous mutation in group IIA sPLA2 that abrogates expression (Kennedy et al, 1995), we selected human group IIA sPLA2 transgenic mice on this background. Thus, the only group IIA sPLA2 activity in these mice derives from the human transgene. Consistent with a pro-inflammatory contribution from group IIA sPLA2 to human autoimmune arthritis, group IIA sPLA2 transgenic mice display increased clinical (Fig 4C) and histological (Fig 4D) arthritic responses to K/BxN serum transfer.

**Group X sPLA2 deficiency does not impact arthritis**

Group V sPLA2 and group X sPLA2 are unique among mammalian sPLA2s 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 sPLA2. We found no contribution of group X sPLA2 in K/BxN serum-induced erosive arthritis (Supporting Information Fig S2), further confirming separate and distinct functions of individual sPLA2 enzymes in arthritis pathophysiology.

**Group V sPLA2 stimulates phagocytic uptake of immune complexes**

Several of the known activities of group V sPLA2 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 sPLA2 deficient macrophages (Balestrieri et al, 2006, 2009). We therefore hypothesized that group V sPLA2-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 sPLA2 (Fig 5A).

To assess a homologous activity for human group V sPLA2 and thereby extend the relevance of our observations to human autoimmune inflammatory disease, we added exogenous human group V sPLA2 to leukocytes present in SF from inflamed joints of RA patients and monitored phagocytosis of IgG immune complexes by CD14+ macrophages (Balestrieri et al, 2006, 2009). Consistent with our murine observations, we found that human group V sPLA2 can trigger phagocytosis of IgG immune complexes in this population of cells that are abundant in the diseased joint fluid (Fig 5B).

**Group V sPLA2 promotes immune complex phagocytosis via cysteinyl leukotriene generation**

We further examined the mechanisms by which group V sPLA2 promotes phagocytic uptake of IgG-containing immune complexes. Previous studies have demonstrated that group V sPLA2 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 sPLA2 to generate an enzymatically inactive protein (group V sPLA2-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 sPLA2, we endeavoured to identify which lipid(s) promotes immune complex phagocytosis. Once released from the phospholipidic bilayer by a PLA2, arachidonic acid can be metabolized into several classes of biologically active lipids including prostanoids, leukotrienes, lipoxins, resolvins and others via the cyclooxygenase (COX) or lipxygenase (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 sPLA2 in human SF CD14+ monocyte/macrophage cells (Fig 5C).

To define which 5-LO-dependent eicosanoid mediates group V sPLA2 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 sPLA2 to promote immune complex uptake by phagocytes. Examination of supernatants from RA SF leukocytes or peripheral blood mononuclear cells (PBMC) treated with group V sPLA2 demonstrated significant stimulation of cysLT generation by these populations (Figs 5D and 6D). In stark contrast, group IIA sPLA2 and enzymatically inactive group V sPLA2-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 LTC4 and LTD4 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 sPLA2 of immune complex phagocytosis by CD14+ cells (Fig 5G). In sum, our findings point to a novel pathway in which group V sPLA2 promotes immune complex clearance in monocyte/macrophage cells via stimulating synthesis of CysLTs, which act through CysLTR1 to promote phagocytosis.

**Group V sPLA2 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 sPLA2 impacts immune complex clearance \textit{in vivo}. Consistent with this mechanistic contribution by group V sPLA2 to temper the severity of arthritis, we found significantly more articular immune complexes and C3 deposition in mice lacking group V sPLA2 than their congenic WT littermates (Fig 6A and B).

To assess whether group V sPLA2 impacts the systemic metabolism of immune complexes, we quantified circulating immune complexes in WT and group V sPLA2 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 sPLA2 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 sPLA2 stimulated comparable phagocytosis of immune complexes by human CD14+ cells, whether from the circulation or from SF (Fig S5 Fig S5B, 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 sPLA2 binding, we hypothesized that these cells may block group V sPLA2 activity in the circulation. Indeed, addition of small amounts of erythrocytes to the assays wherein group V sPLA2 was administered to phagocytes potently inhibited group V sPLA2 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 sPLA2 isoforms was an overlapping pro-inflammatory contribution from individual sPLA2 isoforms to inflammatory arthritis. Indeed, group V sPLA2 is potent at releasing arachidonic acid from cell membranes as a substrate for leukotriene synthesis (Kim et al, 2002), and group V sPLA2-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 sPLA2 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 sPLA2 in inflammatory arthritis. Our mechanistic investigation was guided by our recent observation that group V sPLA2 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 sPLA2 and the increase in immune complex deposition in joint tissues of group V sPLA2 null mice are consistent with an impact of group V sPLA2 on the severity of arthritis by modulating immune complex clearance. This hypothesis was further substantiated by the ability of recombinant group V sPLA2 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 sPLA2 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 sPLA2 in autoimmune inflammatory arthritis, they also point to anatomically or context-dependent actions of this enzyme. On the one hand group V sPLA2 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 sPLA2. 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 sPLA2 (Balestrieri et al, 2006). Taken together, an integrated view of these distinct properties is that group V sPLA2 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 sPLA2 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 sPLA2 promotes clearance of immune complexes in vitro.

A. Phagocytosis of immune complexes in vitro by peritoneal macrophages from group V sPLA2-null, group V sPLA2-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 sPLA2 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 sPLA2 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 sPLA2. Group V sPLA2, its mutant H484Q or group IIA sPLA2 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 LTC4 (E) or LTD4 (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 performed in duplicate.
Figure 6. Group V sPLA₂ 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 sPLA₂-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 sPLA₂-null ankle joints.
C. ELISA quantification of circulating immune complexes in sera from group V sPLA₂ null and group V sPLA₂ 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 sPLA₂ 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 sPLA₂ 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 sPLA2 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 sPLA2 did not display overt toxicity and that the pulmonary pathology observed in transgenic mice over-expressing group V sPLA2 (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 sPLA2 to stimulate cysLT generation. Although not evident in all strains, recent mouse studies also demonstrate an impact of group V sPLA2 on vascular inflammation (Bostrom et al, 2007; Boyanovsky et al, 2009). Thus, extensive assessment of toxicity with chronic parenteral administration of recombinant group V sPLA2 remains warranted prior to investigations in humans.

Group V sPLA2 shares proteoglycan-binding properties with group IIA sPLA2, 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 sPLA2, our studies in mice deficient in group IIA sPLA2 confirmed the pro-inflammatory actions of group IIA sPLA2 in arthritis. Furthermore, mice transgenic for the human group IIA sPLA2 had an exaggerated inflammatory response and worse clinical disease. Although group IIA sPLA2 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 sPLA2 awaits clarification, the absence of cysLT release and the lack of phagocytosis stimulation (data not shown) observed with exogenous administration of group IIA sPLA2 to human macrophages points to a distinct mechanism of activity from group V sPLA2. We speculate that differences in the interfacial-binding domains of group V and group IIA sPLA2 may factor prominently in this functional dichotomy. Group IIA sPLA2 prefers membranes rich in anionic phospholipids whereas group V sPLA2 avidly binds phosphatidylcholine (Singer et al, 2002). The external leaflet of mammalian cells is enriched in phosphatidycholine, thus providing an environment suitable for group V sPLA2. As noted above, further insight into the binding and function of group IIA sPLA2 awaits future investigation.

We also studied mice deficient in group X sPLA2 because this enzyme shares several biochemical properties with group V sPLA2 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 sPLA2. Structurally, based on the crystal structure for human group IIA and group X sPLA2, 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 sPLA2 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 sPLA2 isoforms and the unique anti-inflammatory participation of group V sPLA2 in autoantibody-driven arthritis.

Finally, these studies provide important new insight into therapeutic targeting of sPLA2 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 sPLA2 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-Ghobary & 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 sPLA2 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 sPLA2 inhibitor for treatment of RA should be highly selective for group IIA sPLA2. It has been suggested that lack of efficacy of a group IIA sPLA2 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 sPLA2 and group V sPLA2. Together, our observations provide rationale for pursuing two distinct therapeutic approaches targeted at sPLA2: the use of highly selective group IIA sPLA2 inhibitors and administration of recombinant group V sPLA2.

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 \( \mu l \) of SF were used for all assays except 5 \( \mu l \) was used for group IIA sPLA2. Assay buffer (50 mM Tris, pH 7.8, 0.9% NaCl, 0.02% Tween-20, 0.05% NaNO3, filtered through a 0.45 \( \mu m \) membrane) was added to each well to bring the total volume to 100 \( \mu l \). 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 \( \mu l \) 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/c 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 as control. The BALB/c and C57BL/6 were obtained from Jackson Laboratory (Bar Harbor, ME). The transgenic human group IIA sPLA2 null congenic BALB/c mice (Grass et al, 1996) (C57BL/6 background) and the FcR\( \gamma \) 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 disulfide 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 \( \mu m \) 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).

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 A₂ (sPLA₂) 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 sPLA₂ isoforms present in RA synovial fluid (SF) and their in vivo roles in the disease remain unclear.

**RESULTS:**
Several sPLA₂ species are present in the SF in RA. Importantly, we demonstrate an unexpected and novel anti-inflammatory function for group V sPLA₂ in autoimmune arthritis.

**Phagocytosis of immune complexes by human cells**
Human PBMC were isolated by centrifugation of ethylenediaminetraacetic acid anticoagulated human blood obtained from healthy donors on Ficoll-Paque Premium (GE Healthcare) as described by the manufacturer. PBMC were washed in PBS and resuspended in KRP buffer (4 x 10⁶/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 x 10⁶/ml in KRP buffer. Typically, RA SF comprised ~10 x 10⁵/ml leukocytes, 22.9 ± 4% being CD14+.

**CD14 staining**
When used in phagocytosis experiments, CD14 staining was performed. The monocyte/macrophage population was defined by CD14 staining. A minimum of 5000 cells was analysed in each of three 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 purified PBMC and incubated with group V sPLA₂ 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 reagent (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 defined by CD14 staining. A minimum of 5000 cells was analysed in each of six 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-decalcified 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 denotes 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 immunosorbsent 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 cysteinyi leukotrienes synthesis**
CysLTs were measured in supernatants of cells treated with group V sPLA₂, its inactive mutant group V sPLA₂-H48Q and group IIA sPLA₂ (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 ± 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 sPLA2 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 sPLA2 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 Balesstrieri: Generated critical reagent (group IIA sPLA2 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.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflicts of interest.

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