## SCIENTIFIC DATA: HOT SPOT & ITCH RELIEF SHAMPOO
Scientific papers regarding the use of lidocaine and Hydrocortisone as anti-inflammatory indications.

## INTRODUCTION

**T**here are few things as frustrating for a dog as a lesion of the skin called a hot spot. Often, vet refer to them as moist eczema. Hot spots, also known as acute moist dermatitis, are red, itchy, oozing skin infections that initially emerge as circular lesions, usually on the head, over the hip, and along the side of the chest.



Generally, they begin as an itch or irritation to the dog's skin, such as a flea bite or underlying allergy. The irritation causes the dog to lick and chew the skin. It is the dog's licking and chewing that actually causes the hot spot. Hot spots can arise very quickly: a flea bite can dramatically change into a large oozing and painful wound several inches wide in just a few hours.

Hot spots seem more common in dogs with heavy undercoats and/or long hair coats. Humidity and moisture may both play a role, so long-haired or heavy-coated dogs must be dried thoroughly after swimming or after a bath. Dogs with a history of allergies or ear infections, and those who are allowed to become infested with fleas, or those whose grooming needs are not met (i.e., dogs who have a lot of tangles or mats) are all predisposed to this painful condition. And Pyotraumatic dermatitis is an acute, rapidly developing surface bacterial skin infection that occurs as a result of self-inflicted trauma. These lesions are created when the animal licks, chews, scratches and rubs a focal area of skin in response to an itchy (pruritic), painful stimulus. Because the lesions are warm to the touch, they are often called "hot spots."

As the lesion grows, secondary infection from opportunistic bacteria can occur, causing more discomfort and leading the pet to scratch and chew even more. Other names include wet eczema, moist eczema, summer sores, acute moist alderman, acute moist dermatitis, pyo traumatic dermatitis, or acute pyo traumatic dermatitis. As the nickname "summer sores" suggests, hot spots are more common in the summer; however, the ailment can occur at any time of the year. Many pets that develop them have allergies; they are particularly common in pets with flea allergies. However, any sort of irritation to the skin can result in a hot spot.

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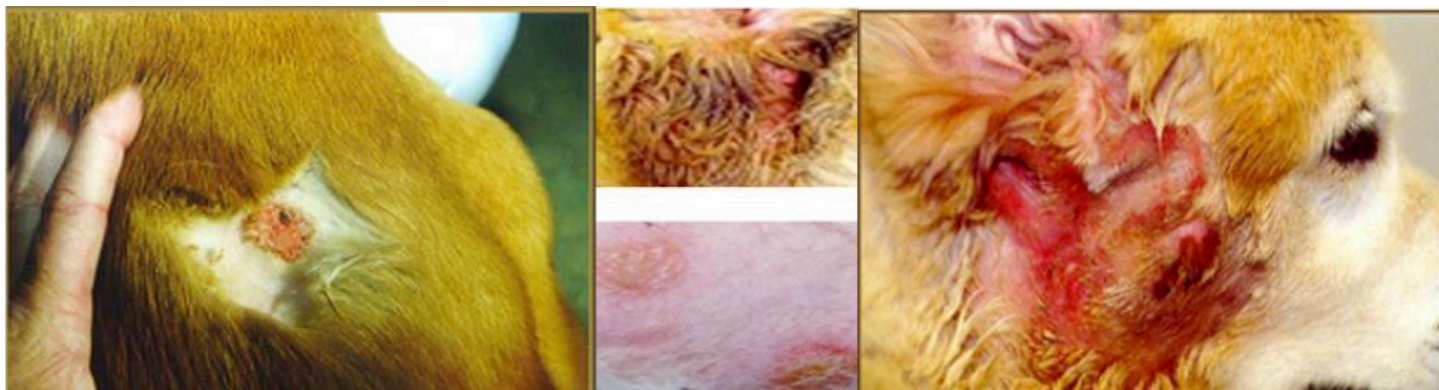
Dogs and cats always have a bacterium near the mouth called Staphylococcus intermedius. This type of Staph, not usually at all contagious to people, is the most common infection found in hot spots.

If your pet has had a history of hot spots that don't seem to heal with regular vet care, be sure she is being tested -- and treated if necessary -- for Methicillin-Resistant Staphylococcus.

Always check with your veterinarian if you are concerned. New information is becoming available all the time. Also, remember that most hot spots are initially caused by fleas. So year-round flea control may help you and your pets avoid these types of infections completely.

If the dog has a hot spot, treatment must be directed at stopping the growth of the hot spot and eliminating the cause. In many dogs the initial cause is fleas or allergies, but lesions below the ear may indicate an ear infection, those near the hip may be related to an anal gland infection, and so on. Whatever the cause, if it can be detected, it must be treated while the hot spot is being treated.

In many locales, it appears that hot spots are less common in the colder temperatures of winter. Many dogs develop several of these lesions over the course of their lives. However, this is generally not a long-term problem if the underlying cause is treated. A lesion may suddenly appear, be treated and be gone in less than a week. Another lesion may suddenly appear later the same summer, the next year or never be seen again on that dog.

Since hot spots can get serious very fast, you should have your dog see a veterinarian as soon as possible. While you are waiting to bring your dog to the veterinarian, you can do some things to make your dog more comfortable. Clip the hair over and up to about an inch surrounding the skin lesion to allow air to get to the inflamed tissue and also to make it easier to treat. Clean the area gently with a non-irritating shampoo. Rinse, and dry thoroughly but gently. Make sure the dog leaves the lesion alone by Place a protective collar on your pet to keep him or her from licking or chewing on the hot spot. Keep an eye on the area to make sure it continues to heal and doesn't worsen or spread.

Preventing this problem is not that difficult if the underlying cause can be found. Many dogs that have repeated problems with hot spots can have the incidence greatly reduced by keeping their hair clipped short during the summer, giving them frequent medicated baths, and reducing the underlying problem through flea control, allergy management, and other preventive measures. The topical Itch Stop contains hydrocortisone to help control itching. Depending on the location of the hot spot, cleaning the ears regularly and expressing the anal glands as needed may also be beneficial. A natural oatmeal bath can also be very helpful.

SYNERGY**LABS**

## Section I:

### THE ART OF SHAMPOOS IN VETERINARY DERMATOLOGY: TREATMENT AND PREVENTION STRATEGIES

Topical therapy (locally acting) is extremely important in the management of many dermatological conditions. Several formulations are available for the prescribing veterinary surgeon: shampoo, lotion, spray, ointment, cream, milk and gel. Choice varies according to the case and must take into account the nature and extent of the lesions, animal's temperament and owner time available. Shampoos are nowadays widely used by veterinary dermatologists.

*Shampoos: What are they, how do they work, how to use them?* A shampoo is an aqueous solution, with added surfactant(s), cleansing agents and various other therapeutic and/or cosmetic agents. Cleaning agents rid the skin surface of debris and help clear the apical pole of hair follicles.

Washing the skin with a topical cleanser should always precede actual topical therapy. Ideally, a shampoo possessing both cleansing and therapeutical properties should be applied twice.

A shampoo can be used in a limited area (eg chin, feet, dorsolumbar, ventral areas), as in humans for the hairy skin, or more commonly all over the body surface of a dog or a cat for treating generalized conditions.

The mechanical effect (elimination of scales and crusts) of the bath is beneficial in all cases. Water rehydrates the stratum corneum although this effect is temporary in the absence of moisturisers.

At the second application, the shampoo must be left on for several minutes, to allow the active ingredients to be properly absorbed and reach adequate levels in the deep cellular layers. This length of time varies between 5 and 15 minutes according to choice of product, concentration, type of base, and the skin condition. The skin should then be rinsed thoroughly, for at least 5 minutes, to prevent irritation and to enable the skin to become adequately hydrated.

The shampoo may be applied several times a week for 2 weeks. The frequency is then reduced to give the longest interval over which treatment is still effective, usually about 1 to 2 weeks.

### Efficacy of shampoo therapy

Clinical improvement is the main criterion to evaluate the efficacy of shampoos (see below for efficacy in specific indications) (2). Their use has increased greatly in North America over the past 25 years, but they have been slow to gain acceptance in Europe (3). However, they are now widely used in the old continent, despite the fact that they were considered as contraindicated and even harmful by many teachers in veterinary schools in the 60's, who recommended "not to wash dogs". This was a mistake and has probably delayed considerably View pictures View other presentations the use of medicated shampoos, which is now considered as being indispensable by the veterinary dermatology community.

The efficacy of shampoos on skin hydration, the surface lipid film and stratum corneum (interesting in case of keratoseborrhoeic disorders) can be evaluated objectively using a variety of techniques : transepidermal water loss (TEWL) measurement, corneocyte counts, measurement of corneal layer thickness, stripping, chemical analysis of lipid film, water content measurement, surface biopsies and corneometry (4-8). In one study (7) corneometry, but not TEWL measurement, was found to give reproducible results. In another study, results from TEWL measurement, corneometry and sebometry were not reproducible and these procedures were therefore deemed to be useless in evaluating effects of topical treatments in the dog (8). Electron microscopy

could perhaps be useful (9).

In recent years, there has been considerable progress in improving topical formulations, especially in prolonging the action of active ingredients applied to the skin. Microencapsulation (multilamellar microvesicles, liposomes, spherulites) increases bioavailability of therapeutic agents and promotes immediate and residual moisturising properties. Active agents are released from liposomes by membrane rupture. Spherulite surfactants are amphiphilic (two antagonistic extremities – one hydrophilic, the other hydrophobic). They unit to form lamellar phases and are arranged in concentric layers according to a specific manufacturing process. They are multilamellar, each membrane acting as a diffusion barrier to reduce loss of active ingredients to the external environment. They can act as a vehicle for a great number of active agents, hydrophilic or hydrophobic (lipophilic), released continuously and progressively at the surface of hairs and skin. This surfactant formulation is very useful in dermatology because it allows hydrophilic, active ingredients access to an oily environment and conversely hydrophobic, active ingredients access to an aquatic medium. The type of surfactant varies. In some cases (cationic surfactants), their charge is positive and spherulites attach preferentially to hairs and skin, whilst in other cases (non-ionic surfactants), the charge is neutral, allowing spherulites to penetrate the deeper skin layers. A study has demonstrated that non-ionic spherulites can penetrate the epidermis, hair follicules, sebaceous glands and dermis (10). The presence of chitosanide reinforces the cationic phase and, by creating a film sheath over the hair, promotes excellent moisturising properties.

A new veterinary formulation (micro-emulsion), with excellent solubility of active ingredients, has recently become available.

The use of shampoos in keratoseborrhoeic disorders (11)

*1 Keratomodulating agents Keratomodulating agents work in two different ways:*
- restoration of normal keratinocyte multiplication and keratinisation. A cytostatic effect is probably exerted on basal cells, thereby reducing their rate of division. Agents working in this way are called keratoplastic (keratoregulating) ;

- elimination of excess corneal layer production, either by increasing desquamation or by reducing intercellular cohesion. Agents that work in this way are called keratolytic.

There are several types of keratomodulating agents. There are also antiseborrhoeic agents which act at the level of the sebaceous gland and its duct (1,12,13).

Salicylic acid is a keratolytic agent. It causes a reduction in skin pH which leads to an increase in:
1) the amount of water that keratin is able to absorb. Stratum corneum hydratation is therefore also increased ;
2) desquamation, via direct effect on intercellular cement.

These actions help soften the corneal layer. Salicylic acid acts synergistically with sulphur, and is often present in small quantities in shampoos. Its efficacy varies with concentration.

Coal tar is a keratoplastic (cytostatic) agent. It reduces nuclear synthesis in the epidermal basal layers (13,14). It is also antiseptic and antipruritic. There are many different sources and varieties of this active agent. Tar is a complex mixture of aromatic hydrocarbons, with many constituents (more than 10,000). It is hard to determine which is (are) responsible for therapeutic effects. Standardisation is therefore difficult, and good quality preparations must be used. Smell and consistency of commercial preparations sometimes make it difficult to use, although deodorised veterinary preparations are now available. Side-effects (e.g. skin drying,

discolouration of pale coats and irritation) have been reported with high concentrations (over 3 %) (1). Its use is contraindicated in the cat.

Sulphur is keratolytic. If forms hydrogen sulphide in the corneal layer and has numerous other, mainly antiseborrhoeic, properties (see below). It is also keratoplastic, due to a direct cytostatic effect and possibly because it interacts with epidermal cysteine to form cystine, an important component of the corneal layer (3,12-14). It is gradually being replaced in topical products by other more effective keratomodulating agents with fewer side-effects (e.g. a rebound increase in scaling).

Selenium disulphide is keratolytic and keratoplastic (reduced epidermal turnover and impaired disulphide bridge formation in keratin). It is also antiseborrhoeic (see below) (3,12,13). It too can cause rebound increase in scaling and sometimes skin irritation.

Ammonium lactate has keratoplastic and keratolytic activity. In the management of human seborrhoea, it has been show to be effective in removing excessive scale by virtue of its keratoplastic activity (15-18). Its mechanisms of action in seborrhoeic disorders have not yet been completely elucidated but it seems to stimulate the living epidermis, correcting defects in keratinocyte multiplication and maturation. This facilitates terminal keratinocyte differentiation, leading to more normal desquamation (19,20). Its properties are useful in seborrhoeic disorders where the ammonium lactate has important moisturising properties (17,18,20). Several clinical studies in man indicate that this substance is very well-tolerated, even when used over prolonged periods (15-19).

2 Antiseborrhoeic agents Antiseborrhoeic agents inhibit or reduce sebum production by the sebaceous glands, and help clear the ducts.

Sulphur (see above) is a classic antiseborrhoeic agent, but is drying and may trigger a rebound effect. It is also antiseptic. It exerts synergistic activity with salicylic acid. This synergism appears optimal when both substances are incorporated into the shampoo in equal concentrations (21).

Selenium disulphide (see above) is antiseborrhoeic, but also has detergent, irritant and drying effects. It is contraindicated in the cat. Benzoyl peroxide, in addition to being antibacterial, is antiseborrhoeic, thanks to sebum hydrolysis and reduced sebaceous gland activity. One study showed that 3% benzoyl peroxide shampoos increase transepidermal water loss and decrease skin surface lipid concentration and corneocyte counts (6). Benzoyl peroxide exerts a follicular flushing action which is very useful when treating comedone disorders and/or follicular hyperkeratosis (3,4,13,22). Sideeffects (irritations, erythematous rash) have been reported especially in concentrations above 5% (4). The skin may also become dry and emollients are therefore always indicated after using this product.

3 Essential fatty acids Various veterinary shampoos have incorporated essential fatty acids for their softening and moisturising properties. One study has demonstrated that in seborrhoeic dogs, abnormal transepidermal water loss could be corrected by applying linoleic acid (23). Some shampoos contain moisturisers : glycerin, lactic acid and fatty acid polyesters. Moisturisers can be stored in multilamellar structures for prolonged release (spherulites), or mono/oligolamellar bodies (liposomes) to ensure hydratation levels are maintained.

4 How to use shampoos in keratoseborrhoeic disorders Certain guidelines are suggested : - long-haired dogs with severe seborrhoeic disorders should be clipped. Clipping leads to more effective application and better distribution of the active ingredient ; - shampoos should initially be applied 2 to 3 times weekly. With time, frequency of application can gradually be reduced ; - cases should be monitored frequently. The therapeutic

agent often needs to be changed following the development of side-effects, rebound effects or change in clinical presentation (e.g. transition from greasy seborrhoea to dry seborrhoea).

IV The use of shampoos in parasitic diseases

Antiparasitic shampoos, ie containing organochlorines, natural pyrethrins or synthetic pyrethroids, are not considered to be as efficacious as antiparasitic rinses and dips (12) and other formulations (sprays, pump-sprays, powders, spot-ons, line-ons, systemic agents), mainly because they are rinsed and cannot act during a sufficient time (2). Scabies, cheyletiellosis, otodectic mange, tick infestation, trombiculosis and pediculosis are relative indications.

Insecticidal shampoos often contain synthetic pyrethroids chosen for their rapid knock-down effect : these are best used as a convenient one-off treatment to rid an animal of a resident flea infestation.

As there is usually little or no residual action once the shampoo is rinsed off, the treated animal is immediately vulnerable to reinfestation by host-seeking fleas. Normally, therefore, shampoos have limited application in the long-term management of flea infestation (pulicosis) and flea allergy dermatitis (24). However, a shampoo containing deltamethrin (0.07%) has been shown recently to maintain a > 90 % antifeeding effect during the hour following challenge for one week (25).

Colloidal oatmeal, an antipruritic agent (see below) is added to bioallethrin , a pyrethroid, in a shampoo to decrease inflammation due to the parasitic infestation.

Benzoyl peroxide shampoos are recommended in the treatment of demodicosis because of their degreasing and follicular flushing effect (3,12).

Many parasitic diseases (e g scabies, cheyletiellosis) and flea allergy dermatitis can cause a keratoseborrhoeic disorder and the affected animals will benefit from application of keratomodulating shampoos (3).

V The use of shampoos in bacterial diseases (pyoderma) Topical therapy is used in canine pyoderma to reduce the cutaneous bacterial population and antibacterial shampoos also remove tissue debris, allowing direct contact of the active ingredient with the organism and promoting drainage (12).

Limited cases of superficial pyoderma can be treated with shampoos alone, particularly if they are used frequently at the beginning (e g every day), then decreasing the frequency of applications depending on the animal's response. However in most cases systemic antibiotics will be administered to ensure a more prompt response, the shampoo playing a supporting role (3). A common indication for long-term use is in the dog that is prone to recurrent folliculitis, either idiopathic or eventually secondary to endocrine or allergic skin disease, even though the pruritus due to the allergic skin disease is controlled. In these situations well tolerated antibacterial shampoos may have a prophylactic effect if used regularly i e every one to two weeks(3,12).

In case of deep pyoderma, clipping is preferable before using shampoos (and soaks). This will prevent the formation of a sealing crust and allow the product to contact the lesions (furuncles, ulcers) (12). In such cases, shampoos should be used very frequently at the initiation of treatment.

Agents commonly included in antibacterial shampoos are chlorhexidine, povidone-iodine, benzoyl peroxide and ethyl lactate.

Chlorhexidine (4,26) is a biguanide antiseptic, very effective against most bacteria (Gram + and -), except some Pseudomonas and Serratia strains. It is bactericidal by action on cytoplasmic membrane which causes

leak of intracellular components. Concentrations vary in shampoos from 0.5 to 4% (diacetate or digluconate). It has a prophylactic effect due to its remanence (27,28). It is well tolerated.

Povidone-iodine is a iodophore which slowly releases iodine to tissues (4,12). The titratable iodine is usually of the order of 0.2 to 0.4 per cent. It is bactericidal and acts in a few seconds at 0.005 % (2). It has also a prophylactic effect due to its remanence (28). It is relatively drying which can be compensated by emollients in shampoos. It can be irritant and staining (4).

Benzoyl peroxide (see above) is metabolized in the skin to benzoic acid and much of its microbiocidal activity probably derives from the lowered skin pH (3). This disrupts microbial cell membranes (3,4). It is in fact an oxidizing agent, which releases nascent oxygen into the skin and produces a series of chemical reactions resulting in permeability changes and rupture of bacterial membranes (4). It has an excellent prophylactic effect, the best one in a comparative study with chlorhexidine, complexed iodine and triclosan (28). It is generally used in concentrations of 2 to 3 %, which are well tolerated but irritation can occur at higher concentrations (erythema, pruritus and pain) (4). In a clinical study done in 1983 in 30 dogs with folliculitis, 61 % of dogs responded well to a 2.5 % benzoyl peroxide shampoo, without concurrent therapy (29). In a comparative study on superficial pyoderma, 70 % of 10 dogs responded well to a 2.5 % benzoyl peroxide shampoo (30).

Ethyl lactate is hydrolysed in the skin to ethanol and lactic acid, thus lowering the skin pH and acting similarly to benzoyl peroxide (3). It is used in concentration of 10 %, which is rarely followed by indesirable side effects (irritation, erythema, pruritus) (4). In a comparative study to benzoyl peroxide, 90 % of 30 dogs with superficial pyoderma responded well to a 10 % ethyl lactate shampoo (30). In a recent study comparing two groups of 10 dogs with superficial pyoderma, it was shown that utilization of a 10 % ethyl lactate shampoo twice weekly reduces the length of systemic antibiotic needed in canine superficial pyoderma (31). Other antibacterial agents used in shampoos are hexachlorophene (not much used because of neurotoxicity), hexetidine (only one product) and triclosan (less effective than benzoyl peroxide and chlorhexidine in a comparative study) (28). In a recent shampoo formulated specifically for canine atopy, piroctone olamine (widely used in human shampoos) has been added for its antibacterial and anti-yeast properties (see below).

VI The use of shampoos in fungal diseases Antimycotic shampoos are used as adjunctive therapy for dermatophytosis and Malassezia dermatitis.

They limit contagiosity in case of dermatophytosis but are not effective in treating it when used alone (32). Non antifungal shampoos or shampoos with insufficient antifungal properties can disseminate spores (33). However, keratomodulating shampoos are used before antifungal topical therapy when there is a keratoseborrhoeic disorder and they are then beneficial in removing infected scales and crusts. In an in vitro study a ketoconazole shampoo was effective to inhibit the growth on Dermatophyte Test Medium of Microsporum canis from infected hair, but after more applications than antifungal solutions (enilconazole, lime sulfur, 2% chlorhexidine, povidone iodine) (34). In a review, a miconazole shampoo is considered to be as effective as lime sulfur and enilconazole in treating feline dermatophytosis (33). In a recent study a shampoo containing chlorhexidine (2%) and miconazole (2%) was shown to accelerate the clinical cure but not the mycological cure of cats infected with Microsporum canis and treated with griseofulvin (35).

Topical therapy is an alternative to systemic treatment in Malassezia dermatitis. For extensive lesions antifungal shampoos or lotions are preferable. They can be used with systemic therapy, although there is no formal evidence that the combination is of greater value than systemic treatment alone. Topical therapy alone should not be used as a diagnostic challenge, but it can maintain a remission, thus confirming the diagnosis. Shampoos containing miconazole (2%), chlrohexidine (2 to 4%), a combination of both (2% each), ketoconazole (2%) or a combination of chlorhexidine (2%) and ketoconazole (1%) are the most appropriate (as are rinses such as lime

sulfur and enilconazole) (12,36,37). Selenium sulphide shampoos could be less effective (37).

A study has demonstrated the immediate and residual in vivo antifungal effect of a shampoo containing piroctone olamine against Malassezia pachydermatis (38).

VII The use of shampoos in allergic diseases
All shampoos are likely to remove allergens from the skin, which is supposed to be useful in canine atopic dermatitis. They also help to rehydrate dry skin, which is common in dogs with allergic skin disease. In addition, shampoos with an antipruritic effect can improve the condition of allergic dogs, provided they are used frequently (e.g. twice a week, at least at initiation of therapy). Antipruritic shampoos are considered generally as adjunctive treatments. They are rarely affective as the sole therapy (3,12).

Antipruritic shampoos contain 1% hydrocortisone, 0.01% fluocinolone, 2% diphenhydramine, 1% pramoxine or colloidal oatmeal. A clinical study has demonstrated that shampoos and rinses containing the local anesthetic, pramoxime, are useful (39). The topical fluocinolone shampoos have been shown not to be systematically absorbed in the dog. Controlled studies on efficacy of antipruritic shampoos are lacking (12).

A shampoo specifically designed for canine atopic dermatitis has been recently developped. It contains linoleic acid, mono and oligosaccharides, vitamine E, and piroctone olamine. Linoleic acid can help in restauring the barrier function of the skin (see above) (23), thus limiting the transcutaneous penetration of allergens. In effect, it has been demonstrated that the stratum corneum intercellular lipids are altered in atopic dogs (9). Mono and oligosaccharides are immunomodulator agents, which can inhibit the secretion of proinflammatory cytokines (such as TNF ) and limit the expression of membrane molecules (such ICAM 1). In vitro studies have been done in man (40) and dog (41). Vitamine E is an antioxidant, stabilizes lysosomes, reduces prostaglandin E2 (PGE2) synthesis, and increases interleukin 2 (IL-2) production with resultant anti-inflammatory and immunostimulatory effects (12). Piroctone olamine is an antiseptic agent active on Gram + and Gram - bacteria, dermatophytes and yeast. It is much used in topical formulations in human dermatology against proliferation of Malassezia furfur. The concept of this shampoo is promising since its goal is to provide a therapeutic response to defects potentially occuring in canine atopic dermatitis. Controlled trials are needed to document the clinical efficacy of this product in atopic dogs.

VIII Moisturisers
In every skin disorders, and in particular with dry seborrhoea, there is scope for increasing the humidity of the animal's skin, after shampooing, with a moisturiser. It has been demonstrated that skin hydratation is less in dogs with scaling than in normal dogs (42).

Moisturisers lubricate, rehydrate and soften the skin. In French, they are all, incorrectly, lumped together as emollients. Moisturisers actually consist of true emollients, emulsifiers/emollients, occlusive dressings and rehydrating agents.

They restore an artificial superficial skin film. Diluted in water, they can be massaged into the skin or applied as a lotion. Undiluted, they may be sprayed on after a shampoo. They should not be rinsed off. In Europe only emollients and rehydrating agents are found in veterinary products (an emulsifier/emollient combination exists in North America). Occlusive dressings are neither used nor marketed in the veterinary field due to risk of maceration.

Emollients are composed principally of fatty acid polyesters, vegetable oils, mineral oils (no veterinary formulations available) and lanolin.

Lipid emollients, containing lanolin alcohols, liquid paraffin or mineral oils, were borrowed from human dermatology and are now rarely used. Used as an emulsion in tepid water, they do improve coat condition, but also have a greasing effect, a definite disadvantage. One veterinary lipid emollient containing fatty acid polyesters is marketed in France. Local application of essential fatty acids has also been proposed to soften and rehydrate the skin, and reduce transcutaneous water loss (23). No major occlusive effect is involved, and the effects are probably brought about by the incorporation of essential fatty acids (especially linoleic acid) into stratum corneum ceramides.

Non-lipid emollients have rehydrating and softening properties. They reduce smell and improve coat appearance without the greasing effect. The high molecular weight of their active ingredients and their hygroscopic nature make them effective surface-protecting therapeutic agents. Examples include lactic acid, glycerin, propylene glycol, urea and chitosanide.

Active agents can be combined with moisturisers : colloidal oatmeal extracts and aloe vera for antipruritic activity, and coal tar for keratolytic and keratoplastic activity.

A lotion has been developped to complement the shampoo specifically designed for canine atopic dermatitis. In a fluid emulsion excipient contains mono and oligosaccharides (free and in spherulites), vitamine E and linoleic acid. This lotion can be used in between shampoos.

IX Conclusion Treatment and prevention strategies in veterinary dermatology include often the use of medicated shampoos. The therapeutic plan should be defined on short and long term basis to obtain the best results, to cope with owners compliance and to limit potential side effects (43). Even with the tremendous recent progresses in companion animal dermatology, there is still a certain amount of art as well as science in devising the optimum topical therapy.

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Section II:
Background
Local and volatile anesthetics are widely used for surgery. It is not known whether anesthetics impinge on the orchestrated events in spontaneous resolution of acute inflammation. Here we investigated whether a commonly used local anesthetic (lidocaine) and a widely used inhaled anesthetic (isoflurane) impact the active process of resolution of inflammation.

Methods and Findings
Using murine peritonitis induced by zymosan and a systems approach, we report that lidocaine delayed and blocked key events in resolution of inflammation. Lidocaine inhibited both PMN apoptosis and macrophage uptake of apoptotic PMN, events that contributed to impaired PMN removal from exudates and thereby delayed the onset of resolution of acute inflammation and return to homeostasis. Lidocaine did not alter the levels of specific lipid mediators, including pro-inflammatory leukotriene B4, prostaglandin E2 and anti-inflammatory lipoxin A4, in the cell-free peritoneal lavages. Addition of a lipoxin A4 stable analog, partially rescued lidocaine-delayed resolution of inflammation. To identify protein components underlying lidocaine's actions in resolution, systematic proteomics was carried out using nanospray-liquid chromatography-tandem mass spectrometry. Lidocaine selectively up-regulated pro-inflammatory proteins including S100A8/9 and CRAMP/LL-37, and down-regulated anti-inflammatory and some pro-resolution peptides and proteins including IL-4, IL-13, TGF-â and Galectin-1. In contrast, the volatile anesthetic isoflurane promoted resolution in this system, diminishing the amplitude of PMN infiltration and shortening the resolution interval (Ri)  50%. In addition, isoflurane down-regulated a panel of pro-inflammatory chemokines and cytokines, as well as proteins known to be active in cell migration and chemotaxis (i.e., CRAMP and cofilin-1). The distinct impact of lidocaine and isoflurane on selective molecules may underlie their opposite actions in resolution of inflammation, namely lidocaine delayed the onset of resoluion (Tmax), while isoflurane shortened resolution interval (Ri).

Conclusions
Taken together, both local and volatile anesthetics impact endogenous resolution program(s), altering specific resolution indices and selective cellular/molecular components in inflammation-resolution. Isoflurane enhances whereas lidocaine impairs timely resolution of acute inflammation.

Introduction
Resolution of acute inflammation was widely held to be a passive event [1]. It is now clear that tissue resolution or its return from an inflammatory and/or disease state is an active process involving novel mediators [2], [3]. Non-resolved inflammation can exacerbate tissue injury and may cause functional damage via abscess or scar formation [1]. An emerging body of evidence now indicates that anti-inflammation (i.e. inhibiting the cardinal signs of inflammation [1]) and pro-resolution, namely activating endogenous resolution programs [3] are distinct mechanisms in the control of inflammation [3,and for a recent consensus report, see ref

4]. Classic antiinflammatories are enzyme inhibitors and/or receptor antagonists such as inhibitors of cyclooxygenases (COX) and antagonists for leukotriene (LT) receptors. Resolution agonists, in comparison, are also antiinflammatories, but act by different mechanisms than the classic ones [recently reviewed in ref 5]. Resolution agonists (such as lipoxins), for example, are agonists that not only block neutrophil (PMN) actions [5], but also stimulate non-phlogistic monocyte recruitment [6] and macrophage uptake of apoptotic PMN [7]. Hence resolution agonists have two main mechanisms of actions at the tissue level; they lower the numbers of infiltrating PMN to the inflamed sites and tissues; and they stimulate the active removal of debris and apoptotic PMN from the inflamed sites by non-phlogistic activation of macrophages [5]. Because it is important to study resolution of inflammation as a distinct process, we introduced resolution indices to a) quantitate the overall process; b) access the roles of specific mediators; and c) pinpoint mechanisms of pharmacological interventions in the resolution of inflammation.

To characterize resolution of inflammation in cellular and molecular terms, we established a resolution map and defined the main quantitative indices (Fig. S1A) [8]. These indices chart and take into account (i) themagnitude of PMN tissue infiltration (maximal PMN, max); (ii) the time interval when numbers of PMN reach max within exudates (Tmax); (iii) duration: the time point (T50) when PMN numbers reduce to 50% of max (R50); and (iv) the resolution interval (Ri): the time interval from the maximum PMN point ( max) to the 50% reduction point (R50)

[i.e. T50-Tmax]. Using this set of resolution indices, we demonstrated that endogenous mediators such as resolvins and protectins accelerate resolution as evidenced by initiating the resolution of inflammation at earlier times ( Tmax and T50) and/or shortening the resolution interval ( Ri)[8], [9]. The actions of these pro-resolution mediators sharply contrast those of agents and currently used therapeutics that are inhibitors and "resolution toxic". These drugs/agents have an unwanted impact on resolution such as inhibitors of COX-2 [9], [10] and lipoxygenases (LOX) [9]. Thus, this set of resolution indices can be utilized to evaluate the impact of endogenous mediators as well as potential new therapeutic agents in inflammatory resolution because they reflect the summation of tissue-level events that are multi-level cellular and molecular processes in resolution of inflammation.

Surgery itself initiates an inflammatory response [11], and local anesthetics, both topical and volatile, are widely used during surgery [12]. Some anesthetics (i.e., lidocaine and isoflurane) are reported to reduce inflammatory markers, including cytokines and chemokines [13], [14]. Potential impact of these widely used anesthetics on resolution of inflammation has not been established. The actions of many widely used current drugs in the pharmacopeia on resolution of inflammation remain unknown because their resolution characteristics were not evaluated at the time of their classical development. Appropriate qualified models of resolution were simply not yet available [8], [9].

Here, we report using an unbiased systems approach that the widely used local anesthetic, lidocaine, and a widely used volatile anesthetic, isoflurane, each impact in vivo the resolution of acute inflammation in opposite directions that were quantified using resolution indices. We also characterize their multi-level impact on key cellular and molecular components in resolution of inflammation.

Results
Local anesthetic lidocaine impairs resolution
We first determined whether lidocaine alters cellular infiltration in a self-limited spontaneously resolving murine peritonitis. For these analyses, we used our reported resolution map that was constructed using an unbiased systems approach that combined cell trafficking into inflammatory exudates and mass spectrometry-based proteomics and lipid mediator lipidomics of resolving exudates [8]. Here, a microbial stimulus, the yeast wall

zymosan A, was administered intraperitoneally to initiate inflammation [15], together with lidocaine given concomitantly. Given the inflammation-resolution map as a background terrain, lidocaine was introduced in order to determine if it significantly changed the signature of resolution map and indices in zymosan-

initiated peritonitis. Inflammatory exudates were collected at the indicated time intervals 4–72 h (Fig. 1A). Zymosan alone, as expected, stimulated an acute increase in the total leukocyte numbers (i.e. PMN and mononuclear cells) present in the peritoneal exudates during the initial phase of inflammation (4 h after zymosan, $11.8\pm0.4\times10^6$ leukocytes), with a maximal infiltration at 12 h ($30.0\pm2.5\times10^6$ leukocytes), followed by a decline or resolution as monitored to 72 h. The time course of PMN infiltration followed a similar trend, peaking ($17.5\pm2.5\times10^6$ PMN) at 12 h after zymosan challenge (Fig. 1A). An anesthetic dose of lidocaine, i.e. 0.08% (w/v) [16] administered with zymosan A significantly increased the number of total leukocytes by 49% within exudates at 4 h ($p<0.05$). The increase in exudate PMN was 58% ($p<0.05$). In the mice treated with both lidocaine and zymosan, the numbers of PMN continued to increase after 12 h and reached a maximum at 24 h. As a result, in the presence of lidocaine, the number of PMN in the exudate was significantly increased at this time point ( 60% increase, $p<0.01$). In contrast, the patterns of mononuclear cell infiltrates did not appear to be significantly altered by lidocaine treatment in this time course (4–72 h). Even doses as low as 0.008% (w/v) lidocaine, when given together with zymosan, led to a significant increase in the accumulation of PMN at 24 h ( 75% increase, $p<0.001$). Lidocaine alone without zymosan challenge did not alter peritoneal leukocyte numbers in this 4–24 h interval after administration (Fig. S1B). These results suggested that lidocaine might hamper PMN clearance during the normal spontaneous resolution phase of acute inflammation.
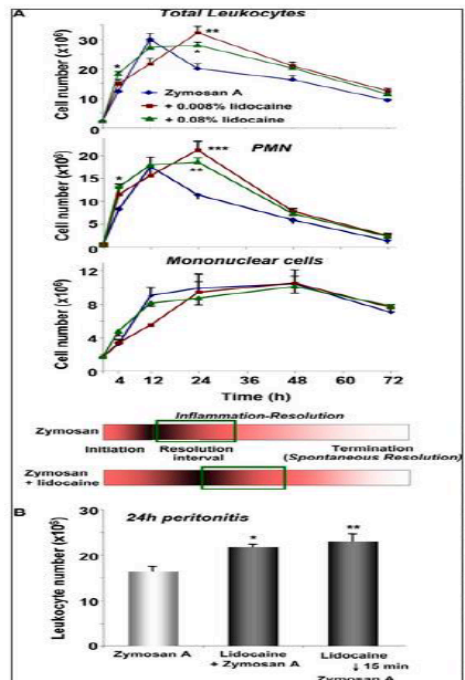


Figure 1
Lidocaine alters leukocyte infiltration during acute inflammation and delays resolution.

## Figure 1

## Lidocaine alters leukocyte infiltration during acute inflammation and delays resolution.

(A) Mice were injected with zymosan A in the absence or presence of lidocaine (0.008% or 0.08%) and peritoneal lavages were collected at indicated time points. Total leukocytes were enumerated by light microscopy, and PMN and mononuclear cells determined by differential leukocyte counting. Results are expressed as the mean±SEM from n = 3–4. *p<0.05, **p<0.01, ***p<0.001 when compared to mice treated with zymosan A alone at the same time points. (B) Mice were injected with lidocaine (0.08%) 15 min prior to injection of zymosan A. Peritoneal lavages were collected at 24 h, and total leukocytes enumerated. Results are expressed as mean±SEM from n = 3. *p<0.05, **p<0.01 when compared to mice treated with zymosan A alone.

As shown in Figures 1A and S1C, in the zymosan-initiated peritonitis, the number of PMN reached a maximum at 12 h. The time intervals between 12 h (Tmax) and 35 h (T50), exudate PMN decreased in number from 17.5×106 PMN ( max; maximal PMN number) to 8.8×106 PMN (R50; essentially 50% reduction of PMN). This period of neutrophilic loss from the exudates is termed the resolution interval (Ri)[8]. In mice treated with zymosan alone, Ri was 23 h (i.e., 12–35 h). When the resolution indices were calculated with lidocaine treatment together with zymosan, it was apparent that both the anesthetic (0.08%) and sub-anesthetic doses (0.008%) of lidocaine increased max and shifted the onset of Ri from 12 h to a later time point (Tmax = 24 h) (see Fig. S1C and vide infra). These results demonstrate that lidocaine directly delayed the spontaneous resolution of zymosan-initiated acute inflammation. Especially, lidocaine increased the dwell time of PMN present within the exudates, possibly blocking the clearance of PMN from the exudates in vivo (see below).

Surgery can induce local inflammation via tissue injury, and lidocaine is usually given before surgery [11],[12]. In order to mimic such a clinical scenario, mice were treated with lidocaine (0.08%) 15 min before initiation of acute inflammation by zymosan. This prior exposure to lidocaine significantly potentiated zymosan-initiated leukocyte infiltration at 24 h by 40% (cf. zymosan alone, p<0.01). This is similar to the results obtained with mice that received lidocaine and zymosan together, which gave a 33% increase in the number of leukocytes present in the exudates when compared to zymosan alone (p<0.05, Fig. 1B). Thus, lidocaine administration, either just before or concomitant with zymosan, caused significant increases in the number of PMN present in exudates in the resolution phase of acute inflammation.

Specialized lipid mediators play a key role in resolution of inflammation [5] with some specifically switched on during the resolution phase to promote resolution [17]. Here, key lipid mediators were monitored in murine exudates, including lipoxin (LX) A4, an anti-inflammatory and pro-resolution mediator, and the pro-inflammatory LTB4 and prostaglandin (PG) E2. In this system, the maximal levels present in cell-free lavages of the exudates of both LTB4 and LXA4 were obtained at 4 h. These subsequently subsided within 24 h (Fig. 2A). Lidocaine did not significantly alter the levels of LXA4, LTB4 or PGE2 present in these cell-free lavages of the peritoneal exudates. Thus, these eicosanoids likely reflect the profile from resident peritoneal cells including macrophages as are less likely to report eicosanoids generated by the infiltrating leukocytes.
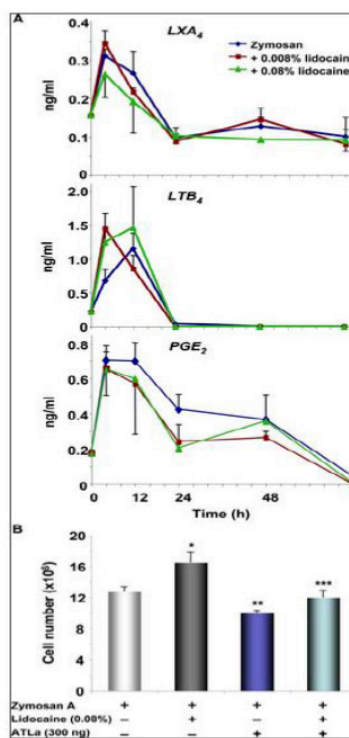
Figure 2

**Lidocaine did not directly alter selective eicosanoid levels in cell-free exudates: LXA$_4$ rescues lidocaine-delayed resolution.**

Lidocaine did not directly alter selective eicosanoid levels in cell-free exudates: LXA4 rescues lidocaine-delayed resolution.

(A) Cell-free lavages from murine peritoneum were collected at indicated time points after zymosan challenge (1 mg/ml). LXA4, LTB4 and PGE2 amounts were determined by ELISA. Results are expressed as the mean±SEM from duplicates of n = 3, and were expressed as amounts (ng/ml). (B) Mice were injected with zymosan A together with lidocaine (0.08%), ATLa (300 ng), or lidocaine plus ATLa. Peritoneal lavages were collected at 24 h, and total leukocytes enumerated. Results are expressed as mean±SEM from n = 3. *p = 0.03 **p = 0.01 when compared to mice treated with zymosan A alone. ***p = 0.04 when compared to mice treated with zymosan A and lidocaine.

Lipoxins are potent agonists for resolution of inflamed tissues by regulating leukocyte infiltration, stimulating macrophage clearance of apoptotic PMN and also their exit via lymphatics [5], [8], [9]. Since LXA4 can rescue inhibitor-imposed lesion with, for example, a selective COX-2 inhibitor [9], we questioned whether these resolution agonists impact leukocyte infiltration in lidocaine-treated mice. At 24 h, lidocaine (0.08%, 0.8 mg) administration increased, while ATLa (a stable analog for aspirin-triggered 15-epi-lipoxin A4, 300 ng, i.p.) decreased exudates cell numbers, when compared with mice treated with zymosan alone. When ATLa was administered along with lidocaine and zymosan, it significantly reduced exudate leukocytes compared to mice received lidocaine and zymosan (p = 0.04) (Fig. 2B). Thus, pro-resolution mediators, such as lipoxins, at much lower doses (by more than 3 log orders) partially rescued the defective resolution of inflammation caused by lidocaine.
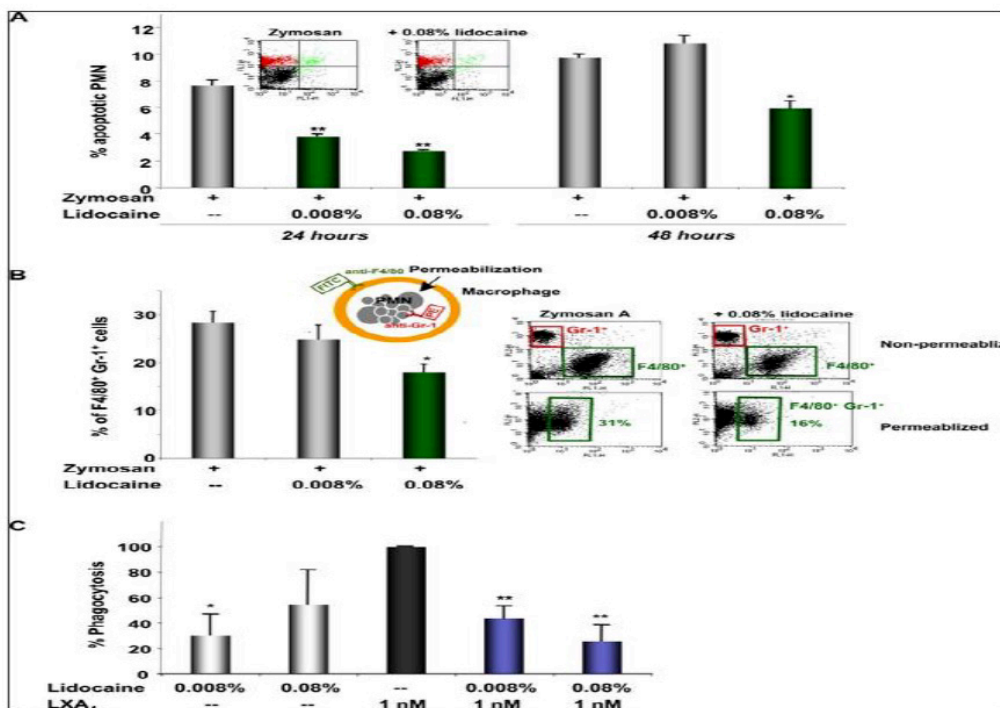
Lidocaine impairs PMN apoptosis and their removal by macrophages

PMN apoptosis and their subsequent removal by macrophages are essential components of resolution at the tissue level [1], [18]. Since lidocaine delayed PMN clearance in the resolution phase, we considered that lidocaine might have an impact on PMN apoptosis. To address this, peritoneal cells were collected at 24 h after zymosan challenge, well within the resolution phase, and labeled with FITC-annexin-V and PE-conjugated anti-Gr-1 Ab, a specific cell surface marker for mouse PMN. Peritoneal cells collected from mice receiving lidocaine (at both 0.08% and 0.008%) together with zymosan showed significantly decreased annexin-V+Gr-1+ cells by 50% and 64%, respectively, indicating reduced PMN apoptosis (Fig. 3A). At 48 h after zymosan challenge, lidocaine at 0.08% also reduced PMN apoptosis  40% (p<0.01).

Lidocaine impairs PMN apoptosis and macrophage ingestion of PMN in vivo and zymosan in vitro.
(A) Apoptosis in vivo. Peritoneal cells were collected at 24 h or 48 h and labeled with FITC-annexin-V and PE-conjugated anti-Gr-1 Ab. The apoptotic PMN (annexin-V+Gr-1+) are expressed as % of total PMN (Gr-1+). Results are the mean±SEM from n = 3–4. *p<0.01, **p<0.001. (B) Phagocytosis in vivo. (right) Representative dot plots of FACS analysis. In the non-permeabilized lavage cells, Gr-1+ represents PMN, and F4/80+ represents macrophages; and in the permeabilized cells, F4/80+Gr-1+ cell population represents macrophages with ingested PMN. (left) Results are expressed as the mean±SEM from n = 3–4, and were expressed as percent of the F4/80+Gr-1+ cells. *p<0.05. (C) Phagocytosis in vitro. Murine peritoneal resident macrophages were incubated with indicated compounds or vehicle alone for 20 min followed by addition of FITC-zymosan at a 10:1 ratio for 30 min. Cells were then quenched and fluorescence determined. Phagocytosis activity in the presence of 1 nM of LXA4 was taken as 100%. Results are expressed as the mean±SEM from n = 3–4, and were expressed as % phagocytosis. *p<0.05, **p<0.01, compared to LXA4 alone.

We next determined whether lidocaine impacts macrophage ingestion of PMNs. To this end, we carried out a phagocytosis-based analysis in vivo (Fig. 3B). Exudate cells were collected at 24 h after zymosan challenge, and macrophages were labeled with the FITC-conjugated anti-F4/80 Ab. This was followed by permeabilization of these cells, allowing labeling of ingested PMN with PE-conjugated anti-Gr-1 Ab. Cells with positive staining of

both F4/80 and Gr-1 were then monitored by FACS analysis. Of interest, cells collected from mice treated with lidocaine (0.08%) together with zymosan showed significantly reduced F4/80+Gr-1+ cells (18.0±1.2%) when compared to those given only zymosan (28.4±1.6%) (p<0.05, Fig. 3B). The low dose of lidocaine (0.008%) also gave decreased F4/80+Gr-1+ cells (24.8±2.1%), albeit not significantly different from mice receiving zymosan alone. These results indicate that clinically used doses of lidocaine inhibit macrophage ingestion of apoptotic PMN in vivo, blocking their removal and resolution.

We also investigated whether lidocaine has a direct impact on isolated macrophages. To this end, we carried out in vitro phagocytosis of zymosan particles. This system represents recognition of microbes by the innate immune system [19]. Recently, we found that pro-resolution mediators such as LXA4 are potent stimulators of macrophage uptake of microbial particles, i.e., opsonized zymosan [9], in addition to stimulating the uptake of apoptotic PMN [7]. Of interest, lidocaine at both doses (0.008% and 0.08%), when added together with LXA4, significantly impaired LXA4-stimulated phagocytosis (Fig. 3C). Thus, lidocaine can be considered "resolution toxic" because impairs key components at the level of tissue resolution, namely PMN apoptosis and macrophage phagocytosis, and blocks the protective action of LXA4.

Lidocaine regulates both anti- and pro-inflammatory proteins: proteomics
Using mass spectrometry-based resolution proteomics, we recently identified several components in inflammatory exudates, including haptoglobin, S100A9 and  1-macroglobulin, that may play active roles in promoting resolution of inflammation [8]. These proteins were identified by peptide mapping of in-gel digested proteins using capillary liquid chromatography-nanospray ion trap tandem mass spectrometry (nanospray-LC-MS-MS) and bioinformatics software (see Methods). Among these, S100A9 was present in exudates within 4 h of initiating inflammation and reached maximum levels at the onset of Ri (12 h). These changes in S100A9 paralleled the time course of PMN infiltration (Fig. 1A) [8]. Both S100A8 and S100A9 are known to be abundant cytosolic proteins in human PMN that can be secreted and exhibit potent actions in inflammatory cell recruitment [20]. Also, S100 proteins belong to a new group of damage-associated molecular pattern proteins and may function as "alarm/danger" signals to propagate inflammation [21]. To determine whether lidocaine impacts these proteins during inflammation-resolution, we carried out temporal-differential analysis of peritoneal exudate proteins collected from zymosan-challenged mice in the presence or absence of the anesthetic dose of lidocaine (0.08%). Two time intervals were selected for analysis: one at 4 h within the early inflammatory phase, and the second at 24 h within the resolution phase, since lidocaine gave the most dramatic impact on PMN infiltration at these two time points (see Fig. 1A). Four hours after zymosan challenge, both S100A8 and S100A9 were significantly increased in the presence of lidocaine (Fig. 4A and Table 1). This increase was verified from mice that received lidocaine together with zymosan by Western blot analysis that also demonstrated an increase in S100A9 proteins in exudates (Fig. 4B). Also S100A9 mRNA levels were higher in these mice compared to mice that received zymosan alone as determined with RT-PCR (Fig. 4B). Thus, it is likely that S100A8/A9 complexes reflect, at least in part, the increases in PMN obtained in mice challenged with lidocaine and zymosan at 4 hours, compared to mice received zymosan alone.
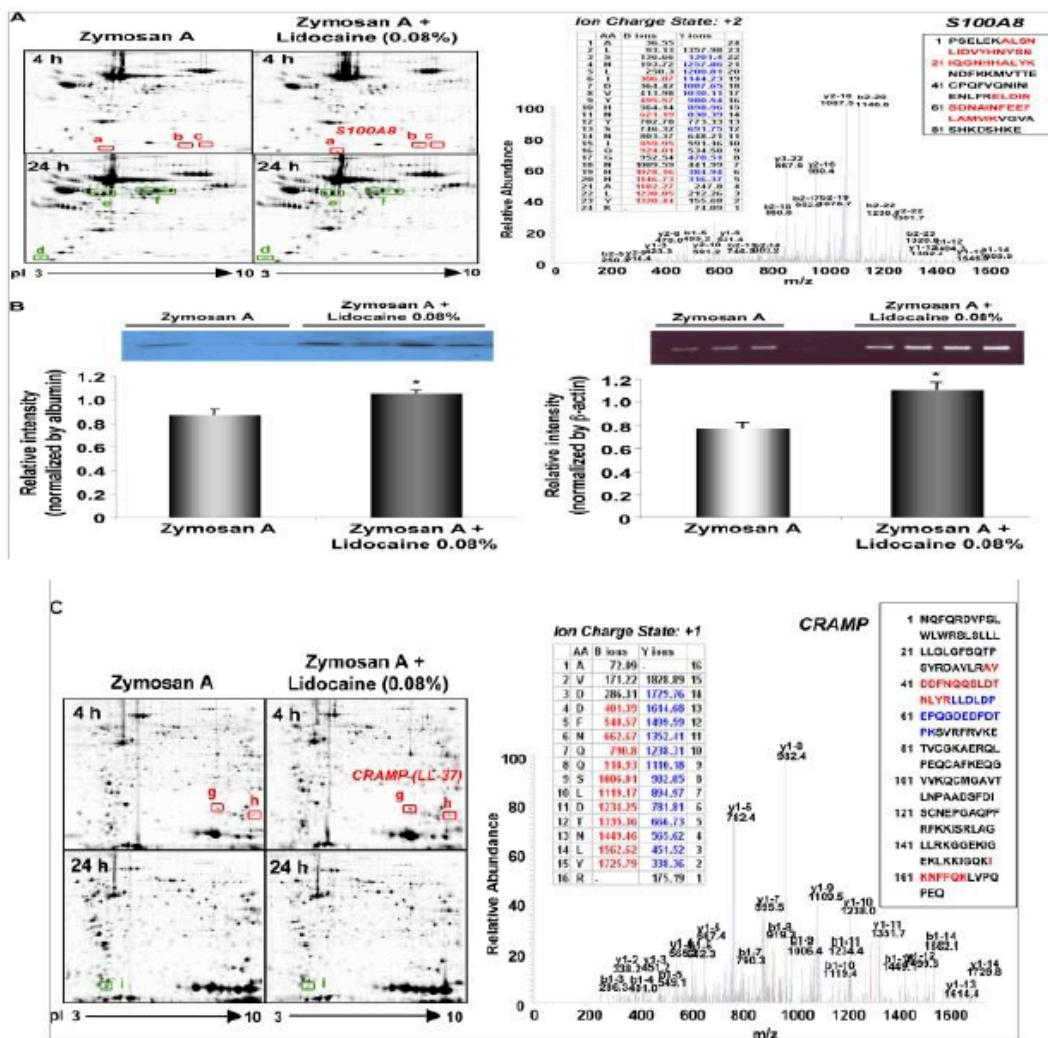
Figure 4

**Lidocaine alters pro- and anti-inflammatory proteins: proteomics and cellular proteins.**

Lidocaine alters pro- and anti-inflammatory proteins: proteomics and cellular proteins.
Mice were injected with zymosan A in the absence or presence of lidocaine. Both lavage fluids (A) and cell pellets (C) were collected at indicated time points and proteins separated by two-dimensional gel electrophoresis. Changes in individual protein levels were measured by image analysis. Selected proteins that display significant differences between treatments are indicated by arrows, and identified by LC/MS/MS and peptide mapping (see Materials and Methods). (B) (Left) S100 A9 protein levels. Supernatants from peritoneal lavages were subjected to Western blot analysis using an anti-S100A9 antibody. Relative intensities of immunoreactive bands were quantitated and normalized by albumin levels using an anti-albumin antibody. Data are expressed as mean±SEM from n = 3–4. *p = 0.02. (Right) S100 A9 mRNA levels. Peritoneal cells were collected and total RNA isolated for RT-PCR analysis using specific primers for mouse S100A9. Relative intensities of RT-PCR products were quantitated and normalized by  -actin message levels. Data are expressed as mean±SEM from n = 3–4. *p<0.01.

| Spot | Protein | Time (h) | # identified peptides | Zymosan A | Zymosan A+Lidocaine | P value |
|---|---|---|---|---|---|---|
| | *Exudate protein* | | | | | |
| a | S100A8 | 4 | 2 | 0.18±0.07 | 0.39±0.08 ( ↑ 117%) | 0.02 |
| b | S100A8 | 4 | 2 | 0.11±0.02 | 0.42±0.10 ( ↑ 282%) | 0.01 |
| c | S100A9 | 4 | 2 | 0.07±0.05 | 0.23±0.06 ( ↑ 229%) | 0.02 |
| d | Apolipoprotein CIII | 24 | 2 | 0.32±0.03 | 0.22±0.02 ( ↓ 31%) | 0.01 |
| e | Fibrinogen γ polypeptide | 24 | 8 | 2.32±0.04 | 1.87±0.17 ( ↓ 20%) | 0.01 |
| f | Fibrinogen β polypeptide | 24 | 16 | 3.97±0.36 | 2.59±0.36( ↓ 35%) | 0.01 |
| | *Cellular protein* | | | | | |
| g | CRAMP (LL-37) | 4 | 2 | 0.27±0.08 | 0.41±0.04 ( ↑ 52%) | 0.05 |
| h | CRAMP (LL-37) | 4 | 3 | 0.14±0.06 | 0.29±0.05 ( ↑ 107%) | 0.03 |
| i | Galectin 1 | 24 | 2 | 0.49±0.06 | 0.23±0.08 ( ↓ 53%) | 0.01 |

The proteins were identified by mass spectrometry (see Materials and Methods).
doi:10.1371/journal.pone.0001879.t001

## Table 1
## Exudate and cellular proteins regulated by lidocaine during inflammation-resolution

We also determined, in parallel with exudate cells, changes in cell-associated proteins from these mice. As shown in Figure 4C, lidocaine together with zymosan at 4 h gave significant up-regulation of several selective proteins compared to zymosan-challenged mice. Among them, CRAMP (cathelin-related anti-microbial peptide), the mouse homolog of anti-microbial protein LL-37, was increased approximately two-fold (Table 1). CRAMP is also a documented chemotactic factor for PMN, monocytes, mast cells, and T cells [22]. Thus, exudate CRAMP/LL-37 may also contribute to increased PMN numbers obtained at 4 h in lidocaine-treated mice (Fig. 1A). Moreover, we found that, within resolution at 24 h, lidocaine down-regulated galectin-1 50% (Fig. 4C and Table 1). Galectin-1 inhibits PMN migration during PMN-endothelial interactions in vitro and in vivo [23]. In addition, Galectin-1 prolongs exposure of phosphatidylserine on the surface of leukocytes, suggesting a role in promoting PMN clearance [24]. Therefore, decreases in Galectin-1 levels from lidocaine-treated mice during resolution (i.e. 24 h) might also contribute to delayed PMN clearance, resulting in increased PMN dwell times in exudates (Fig. 1A).

Lidocaine impacts chemical mediators in exudates
Production of both pro-inflammatory (e.g. IL-1 , IL-6, IL-12, TNF- ) and anti-inflammatory (e.g. IL-4, IL-10, and IL-13) cytokines is essential in the control of inflammation [25]. Here, we monitored a panel of chemokines and cytokines in exudates to assess whether lidocaine specifically regulates their levels. At 4 h after zymosan challenge, most cytokines and chemokines were dramatically up-regulated compared to naïve mice (Fig. 5A). Of interest, both 0.08% and 0.008% lidocaine gave similar results at 4 h, with significant, preferential reduction of anti-inflammatory cytokines, including IL-4, IL-10 and IL-13 (Fig. 5B). In addition, anesthetic dose of lidocaine decreased pro-inflammatory KC (the murine homolog of human IL-8) without significant changes in other pro-inflammatory cytokines and chemokines in the exudates, suggesting that lidocaine acts at several levels in acute inflammation, overall reducing what is coined the "cytokine/chemokine storm" observed in the early inflammatory response (4 h)
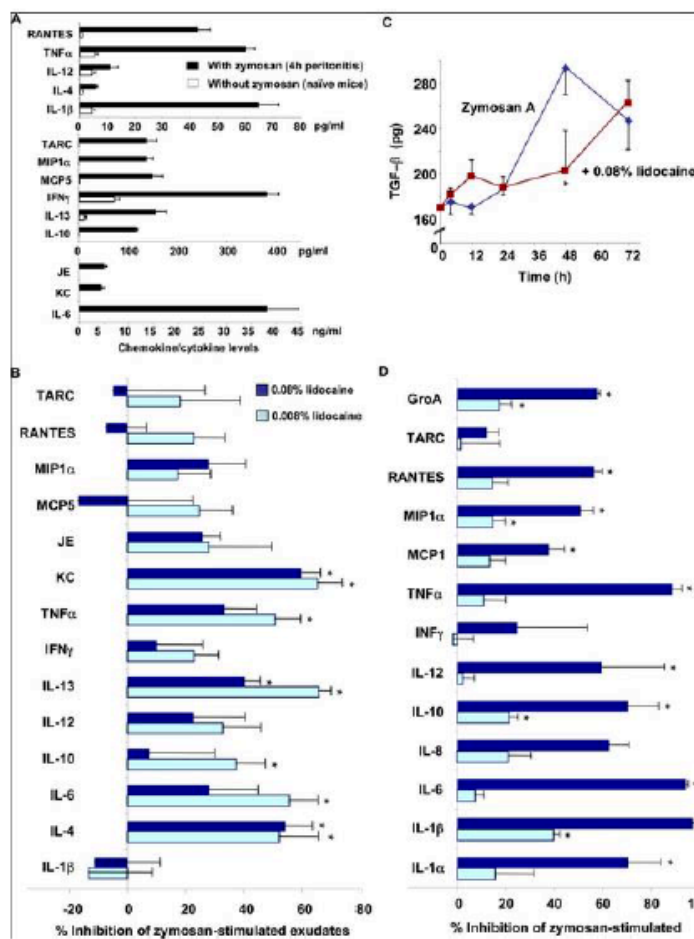
(Fig. 5A). In murine peritoneal exudates, lidocaine initially reduced the levels of most of the chemokines and cytokines induced by zymosan at 4 h, but increased their levels by 12 h (Table 2). Calculation of the ratios between pro- and anti-inflammatory cytokines (TNF- /IL-10 or IL-6/IL-10) indicated that lidocaine increased these ratios in the resolution phase (12 and 24 h after zymosan A injection) (Table 3). These changes in ratios (i.e. pro-/anti-inflammatory cytokines) likely contribute to the increased numbers of PMN present in the resolution phase (i.e. 12-24 h), compared to the mice not treated with lidocaine. We also found that lidocaine (0.08%) significantly decreased exudate levels of TGF-  at the late resolution phase, 48 h after zymosan challenge (Fig. 5C). It is likely that the decreased levels of TGF-  contributed to impaired macrophage phagocytosis in lidocaine-treated mice (Fig. 3B), that can lead to delayed PMN clearance and their increased dwell time. The impact of lidocaine was also evaluated in human whole blood ex vivo to access whether the murine system reflects human tissue events. The anesthetic dose of lidocaine (0.08%) significantly diminished the levels of a panel of chemokines and cytokines in zymosan-stimulated human whole blood (Fig. 5D).

| Time | 4 h | | 12 h | |
|---|---|---|---|---|
| Lidocaine | 0.008% | 0.08% | 0.008% | 0.08% |
| *Pro-inflammatory cytokines* | | | | |
| IL-1β | + | + | ++ | − − |
| IL-6 | − − − [*] | − − | +++ | + |
| IL-12 | − − | − − | − − − | − − − |
| IFN-γ | − | − | ++ | − |
| TNF-α | − − − [*] | − − | +++ [*] | + |
| KC | − − − [*] | − − − [*] | +++ | + |
| JE | − − | − − | +++ [*] | ++ |
| MCP5 | − − | + | ++ [*] | ++ |
| MIP-1α | − | − − | + | − |
| RANTES | − − | + | + | Ø |
| TARC | − | Ø | +++ [*] | − |
| *Anti-inflammatory cytokines* | | | | |
| IL-4 | − − − [*] | − − − [*] | +++ | +++ |
| IL-10 | − − [*] | Ø | +++ | + |
| IL-13 | − − − [*] | − − − [*] | +++ [*] | + |
| TGF-β | Ø | Ø | Ø | Ø |

Mice were administered 1 mg zymosan by intraperitoneal injection in the absence or presence of lidocaine. Peritoneal lavages were obtained at 4 h and leukocytes enumerated. Cell-free fluids were collected and amounts of selected pro- and anti-inflammatory cytokines and chemokines determined by multiplexed sandwich ELISA. Results are expressed as "Ø": no change, "−": 0–20% reduction, "− −": 20–40% reduction, and "− − −": 40–60% reduction; and "+": 0–20% increase, "++": 20–40% increase, and "+++": 40–60% increase of selective cytokine in the presence of lidocaine compared to mice injected with zymosan alone. Results represent mean from n = 3–4. [*] P≤0.05
doi:10.1371/journal.pone.0001879.t002

Mice were administered 1 mg zymosan by intraperitoneal injection in the absence or presence of lidocaine. Peritoneal lavages were obtained at 4 h and leukocytes enumerated. Cell-free fluids were collected and amounts of selected pro-and anti-inflammatory cytokines and chemokines determined by multiplexed sandwich ELISA. Results are expressed as "Ø": no change, "–": 0–20% reduction, "– –": 20–40% reduction, and "– – –": 40–60% reduction; and "+": 0–20% increase, "++": 20–40% increase, and "+++": 40–60% increase of selective cytokine in the presence of lidocaine compared to mice injected with zymosan alone. Results represent mean from n = 3–4. * P≤0.05

| Time | 4 h | 12 h | 24 h |
|---|---|---|---|
| *TNF-α/IL-10* | | | |
| Zymosan A alone | 0.5 | 11.0 | 43.5 |
| +0.008% lidocaine | 0.4 | 12.5 | 66.7 |
| +0.08% lidocaine | 0.4 | 11.0 | ND |
| *IL-6/IL-10* | | | |
| Zymosan A alone | 329.2 | 236.0 | 134.0 |
| +0.008% lidocaine | 232.7 | 301.0 | 177.0 |
| +0.08% lidocaine | 254.8 | 251.0 | ND |

ND, not determined.
doi:10.1371/journal.pone.0001879.t003

Volatile anesthetic isoflurane promotes resolution

Isoflurane 1.4 MAC (minimum alveolar concentration) was administrated over a 2 h period (from 1 h before to 1 h after zymosan challenge) to mimic clinical use. Exudates were collected during inflammation-resolution to determine the potential impact of isoflurane in the resolution maps and indices. Isoflurane significantly reduced zymosan-stimulated leukocyte infiltration at 12, 24 and 48 h (Fig. 6A). When compared to mice that received zymosan alone, at the peak of inflammation, Tmax, isoflurane decreased maximal PMN numbers ( max) from $18.0 \times 106$ to $13.5 \times 106$. In addition, isoflurane dramatically reduced T50 from  34 h to  22 h, thus shortening Ri by >50% from  22 h to  10 h (Fig. 6B). These results contrast with lidocaine's impact on the resolution indices where lidocaine delayed the onset of resolution, i.e. Tmax (Table 4).
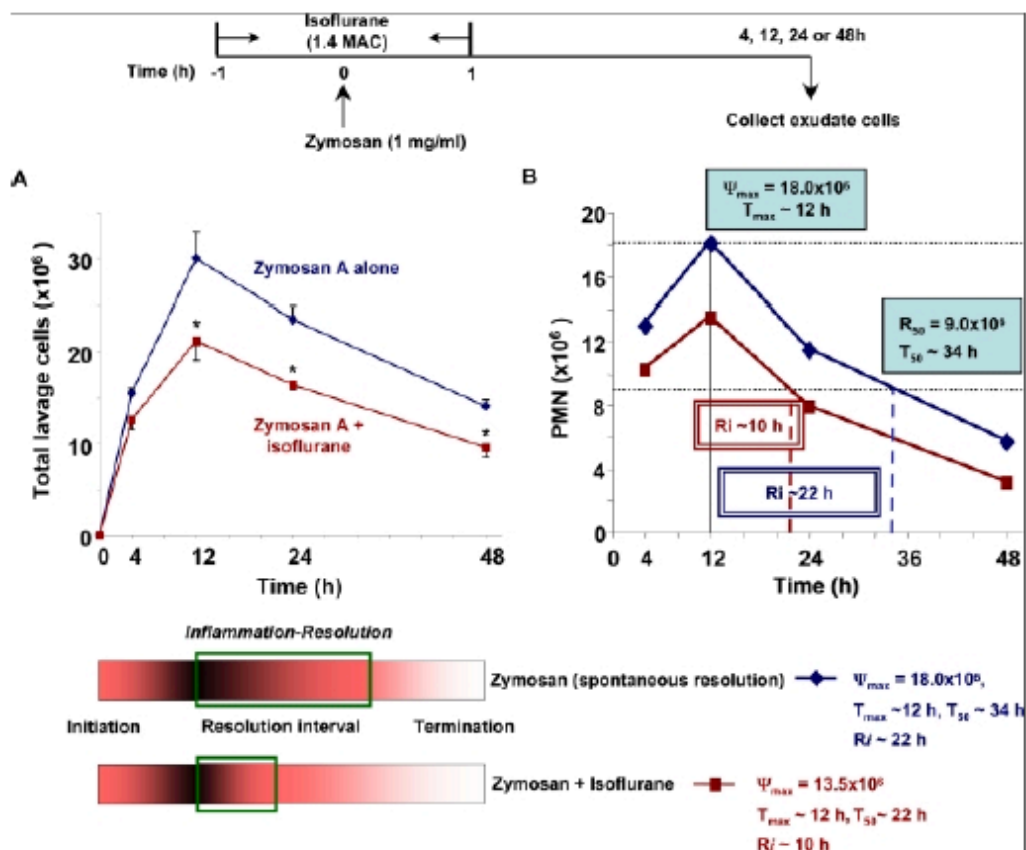


Figure 6

**Volatile anesthetic isoflurane reduces leukocyte infiltration and promote resolution by shortening resolution interval.**

| | $\Psi_{max}$ PMN# (x10$^6$) | $T_{max}$ (hours) | $T_{50}$ (hours) | $R_i$ (hours) |
|---|---|---|---|---|
| Zymosan-initiated peritonitis | 17.5 | 12 | 35 | 23 |
| Zymosan+lidocaine | 18.5 | 24 | 45 | 21 |
| Zymosan-initiated peritonitis | 18.0 | 12 | 34 | 22 |
| Zymosan+isoflurane | 13.5 | 12 | 22 | 10 |

Resolution indices are quantitatively defined as: (i) Magnitude ($\Psi_{max}$, $T_{max}$)–The time point ($T_{max}$), following challenge or injury, when neutrophil numbers in tissues or exudates reach maximum ($\Psi_{max}$); (ii) Duration ($R_{50}$, $T_{50}$)–The time point ($T_{50}$) when the neutrophil numbers reduce to 50% of $\Psi_{max}$ ($R_{50}$); (iii) Resolution Interval ($R_i$)–The time interval from the maximum neutrophil infiltration time point ($\Psi_{max}$) to 50% reduction point ($R_{50}$) [i.e. $T_{50}$–$T_{max}$]. Lidocaine treatment enhanced the magnitude ($\Psi_{max}$) and delayed the onset of resolution ($T_{max}$). In contrast, isoflurane reduced magnitude ($\Psi_{max}$) and shortened resolution interval ($R_i$). See Figure S1 for further details and calculations.
doi:10.1371/journal.pone.0001879.t004

| | $\Psi_{max}$ PMN# (x10$^6$) | $T_{max}$ (hours) | $T_{50}$ (hours) | $R_i$ (hours) |
|---|---|---|---|---|
| Zymosan-initiated peritonitis | 17.5 | 12 | 35 | 23 |
| Zymosan+lidocaine | 18.5 | 24 | 45 | 21 |
| Zymosan-initiated peritonitis | 18.0 | 12 | 34 | 22 |
| Zymosan+isoflurane | 13.5 | 12 | 22 | 10 |

## Table 4

### Regulation of resolution indices: lidocaine vs. isoflurane

Resolution indices are quantitatively defined as: (i) Magnitude ( max, Tmax)–The time point (Tmax), following challenge or injury, when neutrophil numbers in tissues or exudates reach maximum ( max); (ii) Duration (R50, T50)–The time point (T50) when the neutrophil numbers reduce to 50% of max (R50); (iii) Resolution Interval (Ri)–The time interval from the maximum neutrophil infiltration time point ( max) to 50% reduction point (R50) [i.e. T50−Tmax]. Lidocaine treatment enhanced the magnitude ( max) and delayed the onset of resolution (Tmax). In contrast, isoflurane reduced magnitude ( max) and shortened resolution interval (Ri). See Figure S1 for further details and calculations.

Isoflurane specifically regulates key exudate proteins
Since isoflurane gave the most dramatic reduction on zymosan–stimulated PMN infiltration at two time intervals (Fig. 6A), these intervals were selected for exudate proteomic analysis: First, the early resolution phase at 12 h, and second, the late resolution phase at 24 h after zymosan challenge (Fig. 7A). At 12 h after zymosan challenge, we found CRAMP, an anti-microbial protein and chemotactic factor was decreased 2-fold (Table 5) in mice that received 1.4 MAC isoflurane, contrasting with significant increase of CRAMP at 24 h mediated by lidocaine (Table 1). In addition, isoflurane reduced cofilin-1 (Table 5), a major actin-depolymerization factor regulating actin dynamics and generation and maintenance of cell protrusions, key cellular events that are required for migration [26]. Therefore, during resolution of inflammation, isoflurane selectively regulates cellular proteins that are involved in cell migration and chemotaxis (i.e., CRAMP and cofilin-1). By comparison, isoflurane treatment increased SH3 domain-binding glutamic acid-rich like protein (SH3BGRL), which might have anti-oxidative and anti-inflammatory properties [27], [28].
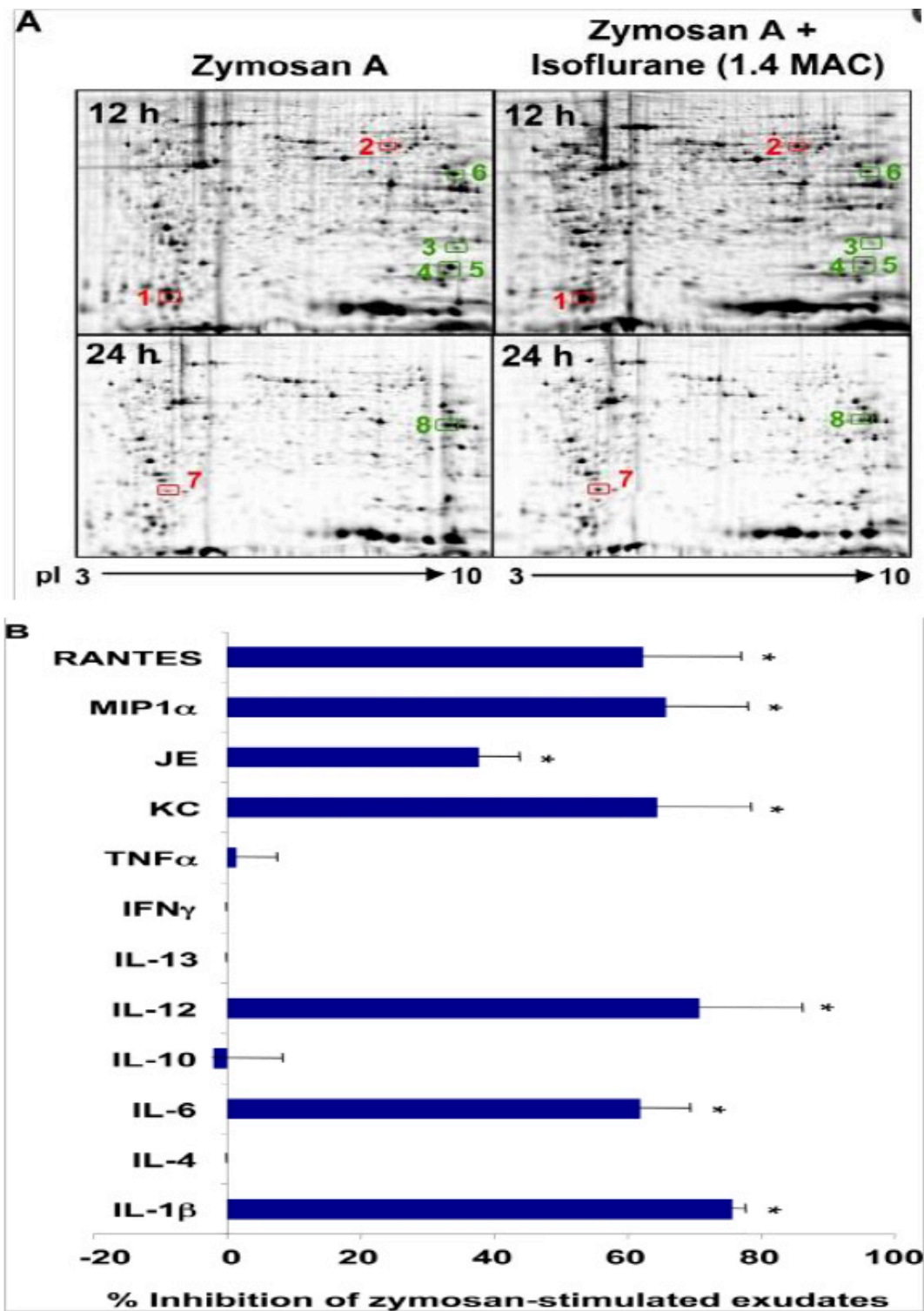
Isoflurane regulates cellular proteins-proteomic analysis.
Mice were administered 1.4 Mac of isoflurane one hour prior to and after injection of zymosan A (1mg/ml, i.p.). (A) The peritoneal lavage cells were collected at indicated time points and proteins separated by two-

dimensional gel electrophoresis. Changes in individual protein levels were measured by image analysis. Selected proteins that display significant differences between treatments are denoted, and were identified by LC/MS/MS and peptide mapping. (B) Peritoneal cell-free lavage fluids were collected. Cytokine and chemokine levels were determined and expressed as percent inhibition of zymosan A-induced cytokine/chemokine levels by isoflurane. *$p < 0.05$ when compared to mice treated with zymosan A alone. For raw values (pg/ml) of these selective cytokines, see Table 6.

| Spot | Protein | Time (h) | # identified peptides | Zymosan A | Zymosan A+Lidocaine | P value |
|------|---------|----------|------------------------|-----------|----------------------|---------|
| Cellular protein | | | | | | |
| 1 | SH3 domain-binding glutamic acid-rich like protein | 12 | 2 | 10442±752 | 18668±4356 (↑79%) | 0.032 |
| 2 | Pyruvate kinase M | 12 | 7 | 1328±200 | 2104±402 (↑58%) | 0.048 |
| 3 | Transgelin-2 | 12 | 5 | 2228±503 | 1129±158 (↓50%) | 0.023 |
| 4 | Destrin (ADF) or Cathelicidin (CRAMP) | 12 | 22 | 4654±190 | 2327±765 (↓50%) | 0.007 |
| 5 | Cofilin-1 | 12 | 2 | 10040±2177 | 4367±953 (↓57%) | 0.014 |
| 6 | Aldolase-1 | 12 | 13 | 6138±1065 | 3730±624 (↓40%) | 0.028 |
| 7 | Neutrophilic granule protein | 24 | 3 | 2139±410 | 4483±318 (↑110%) | 0.001 |
| 8 | Similar to GAPDH | 24 | 2 | 11059±1593 | 4336±1632 (↓61%) | 0.007 |

The proteins were identified by mass spectrometry (see Materials and Methods).
doi:10.1371/journal.pone.0001879.t005

| Spot | Protein | Time (h) | # identified peptides | Zymosan A | Zymosan A+Lidocaine | P value |
|------|---------|----------|------------------------|-----------|----------------------|---------|
| Cellular protein | | | | | | |
| 1 | SH3 domain-binding glutamic acid-rich like protein | 12 | 2 | 10442±752 | 18668±4356 (↑79%) | 0.032 |
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| 6 | Aldolase-1 | 12 | 13 | 6138±1065 | 3730±624 (↓40%) | 0.028 |
| 7 | Neutrophilic granule protein | 24 | 3 | 2139±410 | 4483±318 (↑110%) | 0.001 |
| 8 | Similar to GAPDH | 24 | 2 | 11059±1593 | 4336±1632 (↓61%) | 0.007 |

We monitored in the exudates a panel of chemokines and cytokines to examine whether isoflurane regulates their levels in vivo. Of interest, isoflurane-treated mice selectively reduced zymosan-stimulated pro-inflammatory cytokine levels (IL-1 , IL-6, IL-12, KC, JE [the mouse homolog of human MCP-1], MIP-1  and Rantes) (Fig. 7B, Tables 6 and and7),7), but did not apparently affect the levels of cytokines IL-4, IL-10 and IL-13 in the early inflammatory phase, 4 h after zymosan challenge (Fig. 7B).

| Treatment | Zymosan A | +Lidocaine (0.08%) | Zymosan A | +Isoflurane (1.4 MAC) |
|---|---|---|---|---|
| Pro-inflammatory cytokines (pg/ml) | | | | |
| IL-1$\beta$ | 65.0±7.4 | 72.3±14.8 | 170.0±30.4 | 41.4±3.5* |
| IL-6 | 38183.7±6291.8 | 27391.4±6397.5 | 65093.3±12314.0 | 24808.3±4953.4* |
| IL-12 | 11.2±2.7 | 8.6±2.0 | 9.1±1.6 | 2.7±1.4* |
| TNF-$\alpha$ | 60.5±3.0 | 44.9±6.1 | 128.6±10.6 | 126.8±8.0 |
| KC | 4489.0±553.7 | 1808.5±306.5* | 6311.7±961.5 | 2243.1±890.0* |
| JE | 5074.3±523.3 | 3765.3±319.6 | 8518.3±802.0 | 5308.3±535.4* |
| MIP-1$\alpha$ | 137.0±13.5 | 98.5±17.6 | 173.5±21.9 | 59.4±21.5* |
| RANTES | 42.8±4.7 | 45.9±5.9 | 31.7±5.8 | 11.9±4.7* |

*Significant inhibition of zymosan-stimulated cytokine/chemokine levels

| Treatment | Zymosan A | +Lidocaine (0.08%) | Zymosan A | +Isoflurane (1.4 MAC) |
|---|---|---|---|---|
| Pro-inflammatory cytokines (pg/ml) | | | | |
| IL-1$\beta$ | 65.0±7.4 | 72.3±14.8 | 170.0±30.4 | 41.4±3.5* |
| IL-6 | 38183.7±6291.8 | 27391.4±6397.5 | 65093.3±12314.0 | 24808.3±4953.4* |
| IL-12 | 11.2±2.7 | 8.6±2.0 | 9.1±1.6 | 2.7±1.4* |
| TNF-$\alpha$ | 60.5±3.0 | 44.9±6.1 | 128.6±10.6 | 126.8±8.0 |
| KC | 4489.0±553.7 | 1808.5±306.5* | 6311.7±961.5 | 2243.1±890.0* |
| JE | 5074.3±523.3 | 3765.3±319.6 | 8518.3±802.0 | 5308.3±535.4* |
| MIP-1$\alpha$ | 137.0±13.5 | 98.5±17.6 | 173.5±21.9 | 59.4±21.5* |
| RANTES | 42.8±4.7 | 45.9±5.9 | 31.7±5.8 | 11.9±4.7* |

*Significant inhibition of zymosan-stimulated cytokine/chemokine levels

Table 6

**Regulation of cytokines: lidocaine vs. isoflurane (4h after zymosan challenge)**

| Treatment | +Lidocaine (0.08%) | +Isoflurane (1.4 MAC) |
|---|---|---|
| Pro-inflammatory cytokines (% inhibition by anesthetics) | | |
| IL-1$\beta$ | – | ↓ 75.6% * |
| IL-6 | – | ↓ 61.9% * |
| IL-12 | – | ↓ 70.8% * |
| TNF-$\alpha$ | – | – |
| KC | ↓ 60.7% * | ↓ 64.5% * |
| JE | – | ↓ 27.7% * |
| MIP-1$\alpha$ | – | ↓ 65.8% * |
| RANTES | – | ↓ 62.3% * |

↓ * Significant inhibition of zymosan-stimulated cytokine/chemokine levels
– No significant change
doi:10.1371/journal.pone.0001879.t007

Table 7

**Regulation of cytokines: lidocaine vs. isoflurane (% inhibition)**

Discussion

A systems approach to mapping the resolution of acute inflammation demonstrated that resolution is an active process [2], [3] and a new terrain of cellular and molecular processes directed toward returning the tissue to homeostasis [8], [29]. Using this differential-temporal and quantitative systems approach to analyze inflammation and its spontaneous resolution, we identified, for the first time, in the present report that widely used anesthetics impact the resolution of acute inflammation. Lidocaine, the first amino amide-type local anesthetic is well appreciated to affect depolarization in neurons by blocking the fast voltage gated sodium channels on cell membranes [30]. Earlier evidence from in vitro studies indicates that lidocaine influences the immune system by reducing responses such as chemotaxis, microtubule assembly, phagocytosis, release of lysosomal enzymes and superoxide anion generation [31]–[33]. In certain settings, lidocaine can reduce inflammatory responses and protect tissues from local injury [34]. On the other hand, lidocaine worsens renal injury following ischemia-reperfusion by increasing necrosis and local inflammation [35]. In burn wounds, lidocaine increases leukocyte numbers, which suggests an increase in PMN infiltration and/or increased viability of the leukocytes at the burn site [36]. Yet the clinical significance of these observations remains to be established. The results of the present studies demonstrate that lidocaine imposes a molecular lesion in resolution that delays the return to homeostasis. Specifically, lidocaine increases leukocyte accumulation in exudates, impairs the apoptosis of PMN and hampers ingestion of apoptotic PMN by macrophages in vivo. Summation of these multi-level actions in tissues significantly delays resolution of inflammation.

In this context, other currently and widely used therapeutic agents also affect resolution of inflammation. Aspirin, for example, by way of initiating biosynthesis of endogenous lipid mediators (i.e., aspirin-triggered epimer of lipoxin A4 [ATL] and resolvin E1), promotes resolution [4], [9]. Cyclin-dependent kinase and specific ERK1/2 inhibitors, in comparison, also promote resolution of inflammation by enhancing PMN apoptosis [37], [38]. In contrast, COX or LOX inhibitors, by blocking the biosynthesis of key lipid mediators, dramatically impairs resolution [9], [10]. In the peritoneal cell-free lavages, LXA4 appeared in the early inflammatory phase, 4 h after zymosan challenge. PGE2, a signal that can activate the full LXA4-biosynthetic capacity in vivo [17], was present in the peritoneum prior to peritonitis and elevated during the acute inflammatory response. Lidocaine did not alter either the magnitude or time course of LXA4 in a statistically significant fashion compared to the mice given zymosan alone. However, a trend towards reduction was observed at 4, 12 and 48 h. Of interest, when exogenous ATLa (a stable analog of LXA4 and ATL) was given together with lidocaine, it significantly reversed in part lidocaine's delaying effects in the resolution of inflammation. Thus, pro-resolution mediators may have therapeutic potential in settings where sustained inflammation and impaired resolution are components of disease pathophysiology.

Increases in the ratios of IL-6/IL-10 are thought to signify pro- versus anti-inflammatory response. This increase in ratio correlates with the severity of systemic inflammatory response syndrome and injury after trauma [39]. Since results from several studies indicate that the relationship between pro- and anti-inflammatory cytokines influences the severity of sepsis [40], and TNF /IL-10 ratios are used as an indicator for disease severity [41], we calculated the ratios of pro- to anti-inflammatory cytokines (Table 3) and found that lidocaine increased these values in the late phase (12 and 24 h after zymosan). Thus, changes in the balance between pro- and anti-inflammatory cytokines, rather than individual chemokines or specific cytokines, appear to contribute to the observed increases in PMN numbers obtained at 24 h with lidocaine.

It is noteworthy that, during an inflammatory disease state, a complex network of interactions between different cytokines is likely to occur. The timing of cytokine release and the balance between pro- and anti-inflammatory cytokines is likely to contribute to the overall outcome and severity, as both pro- and anti-inflammatory mediators interact in highly specific ways. Along these lines, computational simulations were carried out to address these complex interactions in the setting of acute inflammation as well as simulate

certain disease scenarios and the time course of cytokine levels in mice [42]. This approach may lead to in silico development of new therapeutics and real-time diagnostics.

The volatile anesthetic isoflurane binds to gamma-aminobutyric acid type A (GABAA) receptors, glutamate and glycine receptors, and inhibits conduction in activated potassium channels [30]. It is noteworthy that human peripheral mononuclear cells express several GABAA receptor subunits, and application of GABA reduced formyl peptide (fMLP)-stimulated increases in intracellular Ca2+ levels [43]. Thus, these GABA receptors may play a role in modulating immune responses. Along these lines, isoflurane is known to impact the inflammatory response, reducing inflammation in vivo [14], increasing leukocyte rolling velocities in mesenteric microcirculation [44], and decreasing activation of the L-selectin and 2-integrins CD11a and CD11b involved in these responses [45]. In the present report, we identified specific proteins in inflammatory exudates and cytoskeleton protein cofilin-1 that were reduced by volatile anesthetics and are known to be important in cell migration. In addition, isoflurane treatment increased SH3BGRL. The human homolog of SH3BGRL belongs to the thioredoxin-like protein superfamily [27]. Among them, thioredoxin-1 (TRX) is a small multifunctional protein with antioxidative and redox-regulating functions [27]. Serum TRX levels were elevated in patients with inflammatory bowel disease. Also, TRX significantly ameliorated DSS-induced colitis and colonic inflammation of IL-10 deficient mice [28]. Thus, SH3BGRL and other thioredoxin-like proteins might have anti-inflammatory properties, and contribute to the accelerated resolution in isoflurane-treated mice documented in the present report (Fig. 6). Of interest, LXA4 stimulates IL-10 [9] as well as heme oxygenase-1 [46], [47] and, as indicated in the present report, was able to partially rescue the lidocaine-delayed resolution of inflammation (Fig. 2B).

Isoflurane and lidocaine gave opposite effects in the resolution of acute inflammation, as indicated by their differential impact in the resolution indices (Table 4). This reflects their distinct and selective impact on specific molecules involved in resolution of inflammation. For example, CRAMP protein levels were decreased in mice with isoflurane, contrasting with significant increases in CRAMP at 24 h as evoked by lidocaine, compared with mice given zymosan alone. Thus, it is likely that the reported chemotactic property of CRAMP [22] contributes to the observed opposing actions of isoflurane and lidocaine on peritoneal leukocyte infiltration. Also, isoflurane selectively reduced zymosan-stimulated pro-inflammatory cytokine levels (Fig. 7B and Tables 6 and and7),7), which contrasts the events in mice treated with zymosan and anesthetic dose of lidocaine (0.08%), that significantly reduced anti-inflammatory IL-13 (Fig. 5B). Hence, the changes in these exudates proteins might reflect the opposing actions of isoflurane and lidocaine in resolution of inflammation.

Historically, the phagocytic index was defined in the cellular era and was used to determine the average number of bacteria ingested by phagocyte at single-cell level [48], [49]. By comparison, the resolution indices presented here expand the appreciation of the complexity of phagocytes at the tissue level and account for the summation of multi-level cellular and molecular events during resolution of inflammation. In conclusion, the results of the recent study indicate that the local anesthetic lidocaine delays the onset of resolution. The impact of lidocaine is documented herein at multi-levels in resolution and reflects (i) increased exudate PMNs, (ii) impaired PMN apoptosis as well as their uptake by macrophages, (iii) modulating both pro- and anti-inflammatory proteins, including cytokines and chemokines. Dysregulation of resolution programs by lidocaine may have important unwanted consequences in both immune responses and host defense that were previously unappreciated.

Clinical implications of the present observations might be far-reaching. Every serious surgical intervention unavoidably results in an inflammatory response. Severity of many postoperative surgical complications, particularly infection, are directly related to the degree and length of inflammatory response and resolution of such response during the postoperative period; therefore, hypothetically, accelerating resolution of postoperative inflammation should be helpful in the management of surgical patients during the postoperative

period. Resolution of inflammation would become a resolution of many problems surgical patients face daily. The results of the present study offer new avenues, not only for continued studies in the cellular and molecular markers in resolution of inflammation, but also for future translational and clinical research. Also, combining pro-resolution molecules, such as LXA4 and ATL, together with lidocaine may be a useful strategy to rescue resolution of acute inflammation. In sharp contrast, the volatile anesthetic isoflurane accelerates resolution, shortening resolution interval. Together, these findings demonstrate, for the first time, the direct impact of anesthetics in the resolution of inflammatory challenge and the return of the local tissue to homeostasis.

Materials and Methods

Murine acute inflammation

For lidocaine treatment, male FVB mice (6-8 weeks; Charles River, Wilmington, MA) were administered lidocaine (0.08% or 0.008%) intraperitoneally together with 1 mg/ml zymosan A (i.p.) to evoke peritonitis [8] as in accordance with the Harvard Medical Area Standing Committee on Animals (protocol no. 02570). ATLa (a stable analog of aspirin-triggered LXA4) was prepared by total organic synthesis in the Organic Synthesis Core (P50-DE016191). For isoflurane treatment, mice were administered 1.4 MAC [50] of isoflurane for a 2 h period (from 1h before to 1 h after injection of zymosan, i.p.) (see timeline in Fig. 6). At indicated time points, mice were euthanized with an overdose of isoflurane, and peritoneal exudates were collected by lavaging with 5 ml sterile saline. Exudate cells and supernatants were obtained for analyses described below.

Human whole blood

Venous blood (anticoagulated with 10 U/ml sodium heparin) was collected from healthy non-smoking volunteers who declared not to have taken any drugs for at least two weeks before the experiments. Informed consent was obtained from each volunteer. The protocol was approved by the Brigham and Women's Hospital Institutional Review Board (protocol no. 88-02642, approved 11/26/07). Heparinized whole blood was then incubated with either 0.008% or 0.08% of lidocaine in the presence of zymosan A (100 g/ml) for 4 h, and plasma was collected by centrifugation at 2,000 rpm for 15 min. The amounts of cytokines and chemokines levels were determined by multiplexed sandwich ELISA (SearchLight Proteome Array custom-designed by Pierce Boston Technology Center). Following a standard sandwich ELISA procedure, the entire plate is imaged to capture chemiluminescent signals generated at each spot within each well of the array. The SearchLight CCD Imaging and Analysis System features image analysis software that calculates chemokine/cytokine concentrations (pg/ml) using pre-determined standard curves.

Differential leukocyte counts and FACS analysis

Aliquots of exudate cells were prepared for determination of total and differential leukocyte counts. For determination of cellular composition (PMN vs. mononuclear cells), cells were blocked with anti-mouse CD16/32 blocking Ab (0.5 g/0.5×106 cells) for 5 min and stained (20 min) with FITC-conjugated anti-mouse CD14 and PE-conjugated anti-mouse Ly-6G (0.5 g/0.5×106 cells; clones rmC5-3 and RB6-8C5, respectively, from BD Pharmingen, San Diego, CA). FACS analysis was then carried out.

Apoptosis and phagocytosis

For determining PMN apoptosis in vivo, exudate cells were labeled with FITC-conjugated anti-annexin-V Ab (0.5 g Ab/0.5×106 cells, eBioscience) and PE-conjugated anti-mouse Gr-1 (Ly-6G) Ab (0.5 g Ab/0.5×106 cells, eBioscience) for 20 min. The annexin-V+Gr-1+ PMN population was determined by FACS.

For determining macrophage phagocytosis of apoptotic PMN in vivo, cells were blocked with anti-mouse CD16/32 blocking Ab (0.5 g/0.5×106 cells) for 5 min, stained with FITC-conjugated anti-mouse F4/80 (0.5 g/0.5×106 cells) for 20 min, and then permeabilized with 0.1 % Triton X-100 (100 l, 10 min). Permeabilized cells were then stained with PE-conjugated anti-mouse Ly-6G (0.5 g/0.5×106 cells). The F4/80+Gr-1+ cell population was determined by FACS.

For phagocytosis in vitro, murine peritoneal resident macrophages were collected and plated onto 24-well plates (1×105cells/well) and incubated with lidocaine (0.008% or 0.08%), LXA4 (1 nM; Calbiochem) or both for 20 min. FITC-zymosan (2.5 l/well) was then added to macrophages for 30 min. Supernatant was aspirated and extracellular fluorescence was quenched by adding trypan blue for 1 min. Cells were then washed and intracellular fluorescence was determined by a fluorescent plate reader.

Two-dimensional gel-based proteomics
Two-dimensional gel electrophoresis
Supernatants and cell pellets from peritoneal lavages were collected by centrifugation (15 min, 1,800 rpm) in the presence of protease inhibitors (Roche, Indianapolis, IN). Proteins in the supernatant were desalted by acetone precipitation, and the protein pellet was re-dissolved in a lysis solution containing 8 M urea, 4% w/v CHAPS, 40 mM Tris, and 65 mM dithiothreitol (DTT). The cell pellets were directly solubilized in the same lysis solution through sonication at 4°C. The protein concentrations were measured in duplicate by a Bradford protein assay kit (Bio-Rad, Hercules, CA) in a 96-well plate format using bovine serum albumin as the standard. Supernatant (25 g) or cellular (50 g) proteins from each animal were mixed with 125 L of rehydration buffer containing 8M urea, 2% (w/v) CHAPS, 10 mM DTT, and 0.2% carrier ampholytes (pH 3–10), and then loaded onto nonlinear 7-cm, pH 3-10, IPG strips (Bio-Rad) through passive in-gel rehydration overnight. After iso-electric focusing for 10,000 V-h, the proteins in the IGP strips were reduced with dithiothreitol and alkylated with iodoacetamide. The 2nd dimension separation was then carried out using 10–14% SDS-PAGE (covering MW10 to 200 kDa). Gels were stained with ProteomIQ™ blue dye (Proteome Systems, Woburn, MA), and scanned with a GS-800 densitometer system (Bio-Rad). Image analysis was carried out with PDQuest software (version 8.0) (Bio-Rad). The differentially regulated protein spots were selected based on the normalized spot volumes.

LC-MS-MS proteomics
The selected protein spots were excised and in-gel digested with sequencing grade trypsin (Promega, Madison, WI). Tryptic peptides were loaded onto a 2 g capacity peptide trap (CapTrap; Michrom Bioresources, Auburn, CA) in 0.1% formic acid and 0.05% trifluoroacetic acid and separated by capillary liquid chromatography using a capillary column (75 m×5 cm×3 m; LC Packings, Amsterdam, The Netherlands) at 150 nl/min delivered by an Agilent 1100LC pump (400 l/min) and a flow splitter (Accurate, LC Packings). A mobile phase gradient was run using mobile phase A (2% acetonitrile/0.1% formic acid), and B (80% acetonitrile/0.1% formic acid) from 0–10 min with 0–20% B followed by 10–90 min with 20–60% B. Peptide mass and charge was determined on a ThermoFinnigan Advantage ion-trap mass spectrometer (San Jose, CA) after electrospray ionization using end-coated spray Silicatip tip (ID 75 m, tip ID 15 m, New Objective) held at a spray voltage of 1.8 kV. After acquisition of the peptide parent ion mass, zoom scans and tandem mass spectra of parent peptide ions above a signal threshold of 2×104 were recorded with dynamic exclusion, using Xcalibur 1.3 data acquisition software (ThermoFinnigan).

Protein identification
Proteins were identified by peptide mapping of tryptic peptide tandem mass spectra using TurboSequest (BioWorks 3.1 software, ThermoFinnigan against indexed Swiss-Prot protein database). Protein modifications that were taken into consideration included methionine oxidation and alkylation of cysteine with iodoacetamide. The search results were filtered by Xcorr vs. charge with 1.5 for singly charged ions, 2.0 for doubly charged ions, and 2.5 for triply charged ions. A protein was considered identified when a minimum of two tryptic peptides were matched

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ELISA-peptide and lipid mediators
Aliquots of supernatants were used to quantitate chemokines and cytokines using a SearchLight Mouse Chemokine Array custom designed with Pierce Boston Technology Center. TGF- levels was determined with ELISA using a monoclonal anti-TGF- antibody (R&D Systems, Minneapolis, MN) recognizing the active forms of TGF- (1, 2, and 3). Eicosanoid ELISAs (LTB4, LXA4 and PGE2) were carried out following manufacturer's instructions (Neogen, Lexington, KY).

Western blot:Supernatants from peritoneal lavages were collected and equal amounts of proteins were subjected to SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) microporous membrane by electroblotting. Membranes were blocked in 5% non-fat milk in TBST (0.9% NaCl and 0.05% Tween-20 in 20 mM Tris/HCl, pH 7.4) and probed with a goat anti-mouse S100A9 polyclonal antibody (0.2 g/ml, R&D Systems) for 1 hour. After washing three times with TBST, membrane were incubated with HRP-linked anti-goat IgG (1:5,000 dilution) for 1 h and the immunoreactive bands were developed by incubating with chemiluminescence substrates and visualized by exposure to an X-ray film.

RT-PCR: Murine peritoneal cells were collected, total RNA was isolated using TriZol reagent (GIBCO BRL, Grand Island, NY) and reverse-transcribed followed by polymerase chain reactions (PCR) using HotStar Master mix (Qiagen) (95°C for 15 min, then 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec) with specific primers for mouse S100A9 (sense: 5 -CCCTGACACCCTGAGCA AGAAG-3 and antisense 5 -TTTCCCAGAACAAAGGCCATTGAG-3 ). Relative intensities of RT-PCR products were quantified and normalized by -actin message levels using the public domain NIH image program (developed at the NIH, available on the Internet)

Statistical approaches :All results were calculated and expressed as mean±standard error of mean (mean±SEM). Group comparisons were carried out using one-way ANOVA or Student's t-test where appropriate, with P values <0.05 taken as statistically significant (sufficient to reject the null hypothesis).

Figure S1
(A) Resolution indices: definitions and calculations. The main events in the resolution of acute inflammation can be quantified [8] with the introduction of resolution indices defined as (i) Magnitude ( max, Tmax)–The time point (Tmax), following challenge or injury, when neutrophil numbers in tissues or exudates reach maximum ( max); (ii) Duration (R50, T50)–The time point (T50) when the neutrophil numbers reduce to 50% of max (R50); (iii) Resolution Interval (Ri)–The time interval from the maximum neutrophil infiltration time point ( max) to 50% reduction point (R50) [i.e. T50-Tmax]. For calculating specific resolution indices and further details, see ref. 8. (B) Lidocaine treatment alone. Mice were injected with lidocaine (0.08%) or saline and peritoneal lavages were collected 4 and 24 h after injection. Total leukocytes were enumerated by light microscopy. Results are expressed as the mean of two separate experiments. (C) Resolution Indices calculated with lidocaine. Lidocaine treatment enhances the magnitude ( max) and delays the onset (Tmax) of resolution.

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Footnotes

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