

# Dichotomy in duration and severity of acute inflammatory responses in humans arising from differentially expressed proresolution pathways

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Lipoxins (Lxs) and aspirin-triggered epi-Lxs (15-epi-LxA<sub>4</sub>) act through the ALX/FPRL1 receptor to block leukocyte trafficking, dampen cytokine/chemokine synthesis, and enhance phagocytic clearance of apoptotic leukocytes—key requisites for inflammatory resolution. Although studies using primarily inbred rodents have highlighted resolution as an active event, little is known about the role resolution pathways play in controlling the duration/profile of inflammatory responses in humans. To examine this, we found two types of responders to cantharidin-induced skin blisters in male healthy volunteers: those with immediate leukocyte accumulation and cytokine/chemokine synthesis followed by early resolution and a second group whose inflammation increased gradually over time followed by delayed resolution. In early resolvers, blister 15-epi-LxA<sub>4</sub> and leukocyte ALX were low, but increased as inflammation abated. In contrast, in delayed resolvers, 15-epi-LxA<sub>4</sub> and ALX were high early in the response but waned as inflammation progressed. Elevating 15-epi-LxA<sub>4</sub> in early resolvers using aspirin increased blister leukocyte ALX but reduced cytokines/chemokines as well as polymorphonuclear leukocyte and macrophage numbers. These findings show that two phenotypes exist in humans with respect to inflammation severity/longevity controlled by proresolution mediators, namely 15-epi-LxA<sub>4</sub>. These data have implications for understanding the etiology of chronic inflammation and future directions in antiinflammatory therapy.

eicosanoids | inflammation | leukocytes

Increasing numbers of mediators are being identified that switch inflammation off, supporting the notion that inflammatory resolution is an active process (1–3). Of these proresolution factors, lipoxins (Lxs; e.g., LxA<sub>4</sub>/B<sub>4</sub>) and epilipoxins (e.g., 15-epi-LxA<sub>4</sub>/B<sub>4</sub>) are lipids with antiinflammatory and proresolution properties (4). By signaling through the ALX (FPRL1) receptor (5), Lxs reduce cytokine/chemokine synthesis and polymorphonuclear leukocyte (PMN) trafficking as well as enhance phagocytosis of apoptotic leukocytes, thereby facilitating multiple aspects of the resolution cascade.

Lxs are formed through three distinct transcellular biosynthetic pathways. The first involves platelet/leukocyte interactions in which leukocyte 5-lipoxygenase (LOX) converts arachidonic acid to the epoxide LTA<sub>4</sub>, which is released and further transformed by leukocyte-adherent platelets to LxA<sub>4</sub> via the Lx synthase activity of platelet 12-LOX (6). A second biosynthetic route occurs at the mucosal surfaces by 15-LOX that inserts oxygen into arachidonic acid at carbon 15 to produce 15S-hydroxyicosatetraenoic acid, which is taken up by PMNs and converted by 5-LOX to Lxs (7, 8). In a third pathway, aspirin triggers epimeric forms of Lxs as a result of acetylating the active site of inducible COX-2 in endothelial or epithelial cells. This results not in the inhibition of COX-2, but in the conversion of arachidonic acid to (15R) hydroxyicosatetraenoic acid, which is rapidly metabolized in a transcellular manner by leukocyte 5-LOX to 15-epi-LxA<sub>4</sub> or

B<sub>4</sub> (9). Interestingly, epi-Lxs are also found in humans under aspirin-free conditions possibly derived from either a cytochrome P450 pathway and/or from an endogenous COX-2-acetylating agent capable of generating epi-Lxs in physiological and inflammatory settings (10, 11).

In addition to Lxs, there are a host of other soluble mediators and cells central to resolution whose expression/synthesis occurs at strategic checkpoints throughout inflammation to switch the response off and restore homeostasis (12–17). Although the sequential activation of these proresolution pathways has almost exclusively been elucidated using inbred rodents, little is known about their role in limiting inflammatory responses in humans. We recently found that cardioprotective doses of aspirin (75 mg; [ASA<sup>low</sup>]) inhibits leukocyte trafficking into cantharidin-induced skin blisters in healthy human volunteers (18). A retrospective analyses of those data revealed that ASA<sup>low</sup> worked in 60% of individuals in an inflammation-dependent manner, i.e., the more severe the inflammation in terms of leukocyte accumulation, the greater the antiinflammatory effects of ASA<sup>low</sup>, with the remaining volunteers (40%) not responding to aspirin. The baseline temporal profile of cell trafficking in aspirin responders peaked in an immediate early fashion and resolved within a few days (hereafter called early resolvers; [E<sup>VS</sup>]). Volunteers who were refractory to ASA<sup>low</sup> had a gradual accumulation of cells that failed to clear the blister site, showing signs of delayed resolution (hereafter called delayed resolvers; [D<sup>VS</sup>]). Further analysis showed that interindividual differences in the biosynthesis of endogenous 15-epi-LxA<sub>4</sub> accounted for the differential inflammatory profiles in D<sup>VS</sup> versus E<sup>VS</sup>. Therefore, these data show that acute inflammatory responses in humans follow two distinct profiles controlled, at least in part, by proresolution pathways. Acquired dysregulation of these protective processes at the genetic level or by antiinflammatory agents with hitherto unappreciated resolution-toxic properties (19) may cause ongoing inflammation, tissue injury, or chronic inflammation.

## Results

**Aspirin Reveals Differential Inflammatory Responses in Humans.** We previously found that ASA<sup>low</sup> was antiinflammatory in 26 male healthy human volunteers in whom it dampened leukocyte traf-

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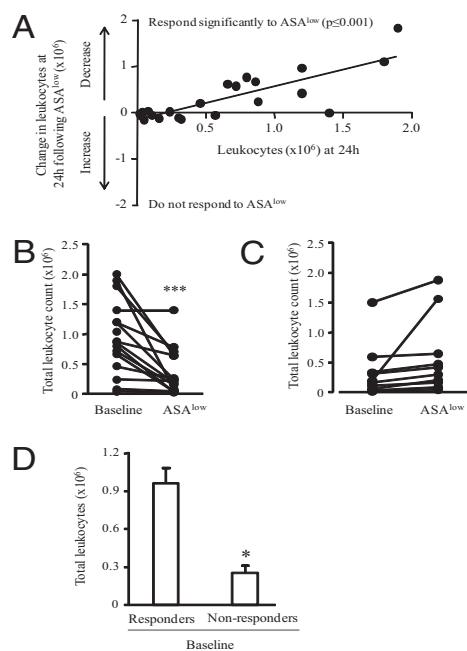
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ficking into cantharidin-induced skin blisters (18). Further analysis of those data revealed that volunteers with the most severe inflammation in terms of blister leukocyte numbers exhibited the greatest antiinflammatory response to ASA<sup>low</sup>. Fig. 1A shows the difference in intrablisters total cell numbers before/after aspirin for each volunteer (y axis) against their corresponding baseline cell numbers on the x axis. Therefore, those above 0 on the y axis (16 volunteers) showed a reduction in cells after aspirin (i.e., responders) whereas those on or below 0 on the y axis showed no change or increased cell numbers, respectively (10 volunteers; i.e., nonresponders). Thus, there are two distinct groups of responders to ASA<sup>low</sup>—those with significantly reduced inflammation after treatment (Fig. 1B) and a second group of 10 volunteers who did not respond to ASA<sup>low</sup> (Fig. 1C). Moreover, there was a significant difference in baseline leukocyte numbers at 24 h between responders and nonresponders (Fig. 1D). These data suggest that, following tissue injury, some subjects display a robust inflammatory response conducive to the antiinflammatory effects of ASA<sup>low</sup> and another group that displays a less severe initial response are refractory to ASA<sup>low</sup>.

**Early Versus Delayed Resolution to Acute Inflammation.** In blisters of volunteers who responded to ASA<sup>low</sup> and who therefore possessed an E<sup>rvs</sup> phenotype, there was a peak in total cells at 24 h following cantharidin (solid line, Fig. 2A), with inflammation resolving by 72 h. In contrast, total cell numbers in cantharidin-induced blisters in the remaining 10 volunteers (D<sup>rvs</sup>; dotted line, Fig. 2A) was

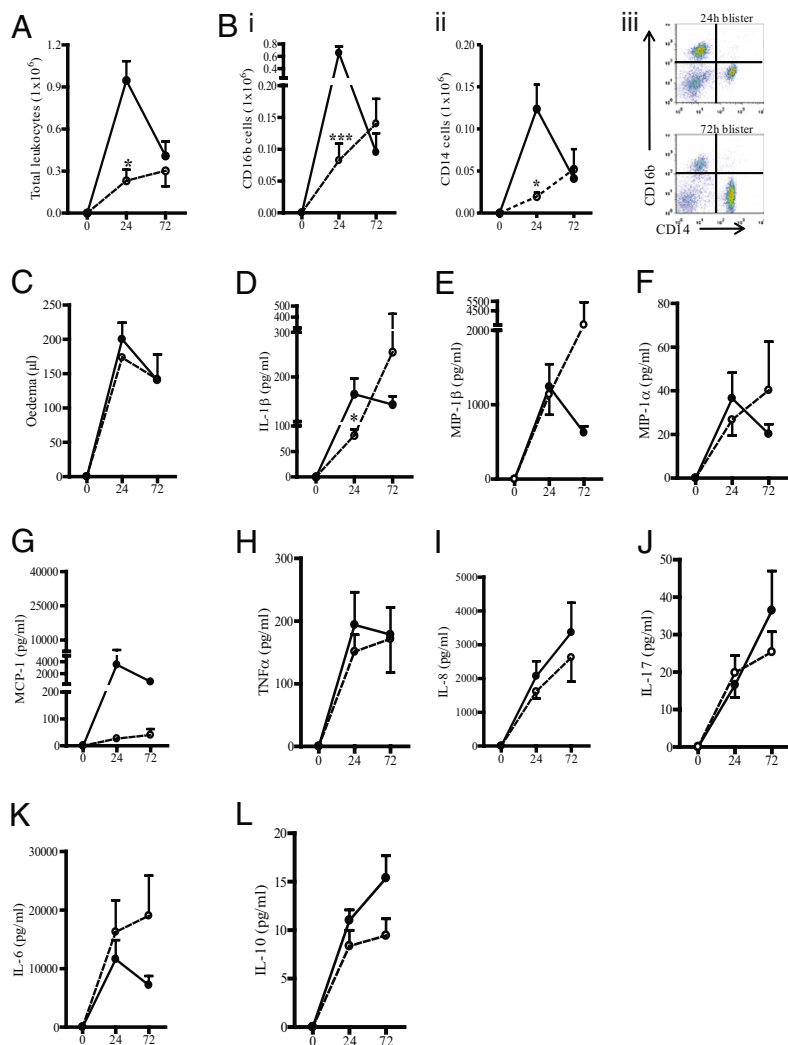


**Fig. 1.** Aspirin reveals differential profiles of acute inflammatory responses in humans. Healthy male volunteers ( $n = 26$ ) between the ages of 25 and 50 y had a cantharidin skin blister elicited on the ventral aspects of their forearm with baseline acute inflammatory responses established 24 h later. Each volunteer was then given ASA<sup>low</sup> (75 mg) for 10 d, followed by creation of another blister. (A) The difference in blister total cell numbers before and after aspirin for each volunteer (y axis) against their corresponding baseline cell numbers on x axis ( $r = -0.81$ ,  $P \leq 0.001$ ). Therefore, those above 0 on the y axis ( $n = 16$ ) showed a reduction in cell after aspirin (i.e., responders) whereas those on or below 0 on the y axis showed no change or increased cell numbers, respectively ( $n = 10$ ; nonresponders). Reduction in blister cell numbers after aspirin was highly significant in responders (B) but not in aspirin nonresponders (C), with differences in baseline inflammation severity between aspirin responders compared with nonresponders also significant (D). Data are presented as mean  $\pm$  SEM; \* $P < 0.05$  and \*\*\* $P < 0.001$ .

significantly lower at 24 h compared with E<sup>rvs</sup> but increased gradually up to 72 h (Fig. 2A). Profiles of CD16b-positive PMNs in Fig. 2B*i* and CD14-positive monocytes/macrophages (Fig. 2B*ii*) followed a similar profile to total cells peaking at 24 h in E<sup>rvs</sup> and declining thereafter but increasing progressively over time in D<sup>rvs</sup>. Representative FACS dot blots are in Fig. 2B*iii*, showing the change from predominantly CD16b-positive PMNs at 24 h to CD14-positive monocytes/macrophages at resolution using E<sup>rvs</sup> as an example. Unlike cells, edema accumulation and clearance followed similar profiles in E<sup>rvs</sup> and D<sup>rvs</sup> (Fig. 2C). Mirroring leukocyte kinetics, levels of IL-1 $\beta$  were significantly higher in E<sup>rvs</sup> versus D<sup>rvs</sup> at 24 h (Fig. 2D), with no such differences found with other cytokine and chemokines (Fig. 2E–L). The results of these experiments reveal two types of responders to acute tissue injury in healthy humans with regard to leukocytes but not edema: the first displayed an early heightened innate immune-mediated response that resolved in an immediate manner, and the second showed a more tempered inflammatory profile that failed to resolve within the timeframe examined (72 h).

**Differences in 15-epi-LxA<sub>4</sub>/ALX Expression Between E<sup>rvs</sup> and D<sup>rvs</sup>.** Inflammation severity is controlled by a diverse range of endogenous counter-regulatory signals. In this current study, by using cantharidin-induced skin blisters, we found no correlation between levels of antiinflammatory IL-10 and leukocyte numbers in E<sup>rvs</sup> and D<sup>rvs</sup>. Similarly, there was no association between leukocyte numbers and levels of prostaglandin E<sub>2</sub>, which exerts inhibitory effects on PMN trafficking via EP2/EP4 receptors by elevating cAMP (20). However, 15-epi-LxA<sub>4</sub> is an endogenous lipid that dampens PMN trafficking (21), proinflammatory cytokine release (ref. 22 and Fig. S1), and facilitates macrophage phagocytosis of apoptotic PMNs (23) through its ALX receptor (5). Levels of blister fluid 15-epi-LxA<sub>4</sub> (Fig. 3A) as well as ALX expression on total cells (Fig. 3B), CD16b-positive PMNs (Fig. 3C), and CD14-positive monocytes/macrophages (Fig. 3D) were significantly lower in E<sup>rvs</sup> (solid line, Fig. 3A) compared with D<sup>rvs</sup> (dotted line, Fig. 3A) at 24 h but were gradually elevated in E<sup>rvs</sup> as inflammation switched off. In contrast, blister fluid 15-epi-LxA<sub>4</sub> (Fig. 3A) and leukocyte ALX expression (Fig. 3B–D) were maximal in D<sup>rvs</sup> at 24 h and showed a trend toward a reduction by 72 h coincident with progressively increased cell influx. Representative FACS histograms in Fig. 3C illustrate increased ALX expression on CD16b-positive PMNs in E<sup>rvs</sup> compared with D<sup>rvs</sup> at 24 h, with similar comparative histograms in Fig. 3D illustrating increased ALX expression on CD14-positive monocytes/macrophages at 24 h. Thus, in groups of volunteers who have an enhanced early response to cantharidin, there are correspondingly low levels of 15epi-LxA<sub>4</sub>/ALX that increased in line with leukocyte clearance. In contrast, D<sup>rvs</sup> have significantly higher 15epi-LxA<sub>4</sub>/ALX than E<sup>rvs</sup> early in the response, but declined as cell numbers increased. Given the antiinflammatory/proresolution properties of epi-Lxs, these data suggest that the dichotomy in response to cantharidin could be caused at least in part by endogenous levels of 15epi-LxA<sub>4</sub> and its receptor expression. The source of 15epi-LxA<sub>4</sub> in humans not taking aspirin is unknown, but mice (not bearing inflammation) given celecoxib have reduced plasma 15epi-LxA<sub>4</sub> whereas levels in animals given SK525A are unchanged (Fig. S2). These data exclude a role for cytochrome 450 in generating endogenous epilipoxins as proposed (11), but suggests the existence of an endogenous factor that acetylates COX-2, resulting in constitutive 15epi-LxA<sub>4</sub> synthesis.

**Modulating 15-epi-LxA<sub>4</sub> and the Inflammatory Response.** As 75 mg aspirin in healthy human volunteers triggers 15epi-LxA<sub>4</sub> synthesis (10) as well as elevates ALX expression (18), we determined whether ASA<sup>low</sup> altered 15epi-LxA<sub>4</sub>/ALX and hence inflammation in E<sup>rvs</sup> and D<sup>rvs</sup>. Ten days of prophylactic ingestion of 75 mg aspirin, taken orally once daily, in E<sup>rvs</sup> (black line, controls; red line, ASA<sup>low</sup>; Fig. 4A) increased blister cell-free exudate levels of 15epi-LxA<sub>4</sub> as well as total leukocyte ALX expression (Fig. 4B–D) coincident with



**Fig. 2.** Dichotomy in the profile of acute inflammatory responses in humans. The inflammatory profile of cantharidin-elicited skin blisters in aspirin responders was charted over time (in h) and compared with nonresponders showing that blister total cell numbers (A), CD16b-positive PMNs (B*i*), and CD14-positive monocytes/macrophages (B*iii*) in aspirin responders (solid line) peaked in an immediate early manner and resolved quicker than in aspirin nonresponders (dotted line); with (B*iii*) a representative FACS dot plot charting the change from primarily CD16b-positive PMNs at 24 h to CD14-positive monocytes/macrophages at resolution in E<sup>rvs</sup>. (C) Blister edema accumulation did not follow this trend. (D–L) Blister fluid exudate proinflammatory cytokines and chemokines with biologically relevant levels broadly mirrored inflammation in both groups. These profiles gave rise to us defining responses as early resolvers (E<sup>rvs</sup>) or delayed resolvers (D<sup>rvs</sup>). Data are presented as mean ± SEM; \**P* < 0.05 and \*\*\**P* < 0.001 represent differences in inflammatory parameters between E<sup>rvs</sup> and D<sup>rvs</sup> at 24 h.

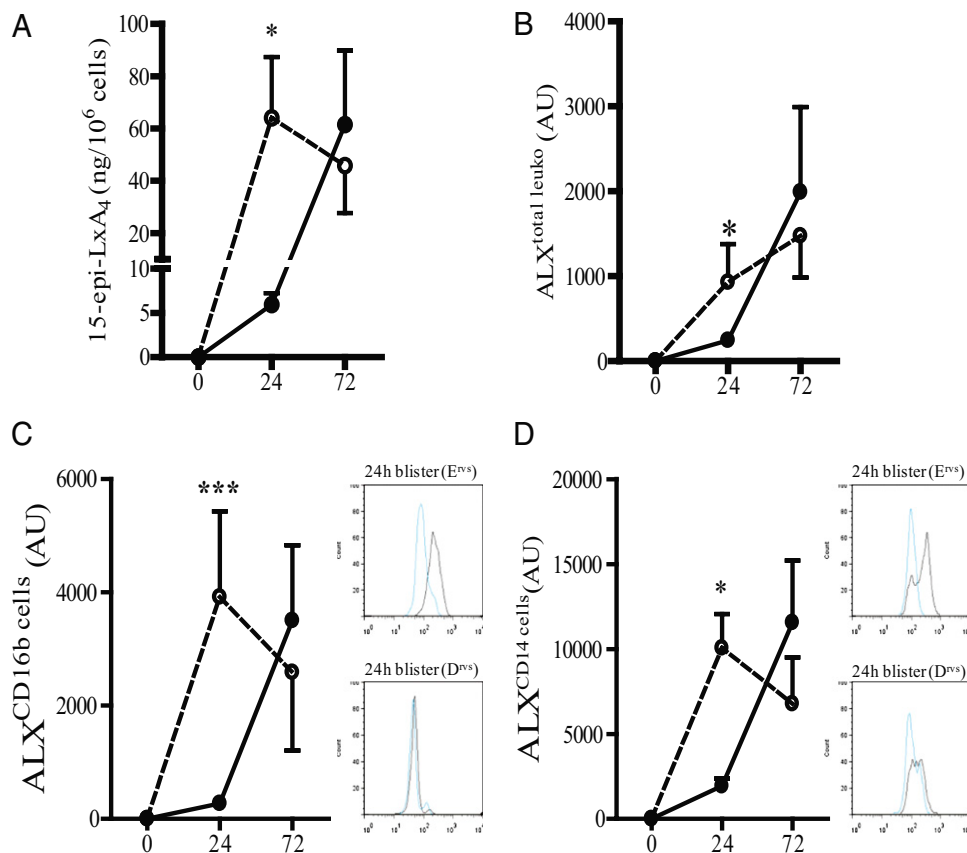
a significant reduction in IL-1β (Fig. 4*E*) and IL-8 (Fig. 4*F*) as well as cell numbers (Fig. 4*G–I*) 24 h following cantharidin. Other cytokines and chemokines were not inhibited (Fig. S3*A–H*). Equivalent treatment of D<sup>rvs</sup> (black line, controls; red line, ASA<sup>low</sup>; Fig. 5*A*) had little effect on 15epi-LxA<sub>4</sub>, leukocyte ALX (Fig. 5*B–D*), IL-1β/IL-8 (Fig. 5*E* and *F* and Fig. S4*A–H*), or cell numbers (Fig. 5*G–I*). These data suggest that 15epi-LxA<sub>4</sub>/ALX are minimal in E<sup>rvs</sup> at 24 h and can be elevated above baseline resulting in a dampening of inflammation. However, in D<sup>rvs</sup> at the same early time point, 15epi-LxA<sub>4</sub>/ALX are maximal and cannot be further elevated. Moreover, it suggests that 15epi-LxA<sub>4</sub> and its receptor are potent endogenous controllers of acute inflammatory responses in humans and their differential expression patterns dictate the severity and longevity of acute inflammatory responses.

## Discussion

At least two distinct inflammatory events can ensue following cantharidin-induced skin blisters in humans. The first is an immediate response typified by rapid cytokine/chemokine generation and leukocyte influx followed by early resolution (i.e., in E<sup>rvs</sup>). The second is a more tempered response characterized by a gradual increase in leukocyte accumulation as well as cytokine/chemokine generation. As inflammation in the latter group did not resolve within the timeframe examined, we called these delayed resolvers (D<sup>rvs</sup>). To gain mechanistic insight into the reasons for these differential responses we found that E<sup>rvs</sup> synthesize low quantities of

15-epi-LxA<sub>4</sub> following injury with levels increasing as inflammation abates. In D<sup>rvs</sup>, synthesis of 15-epi-LxA<sub>4</sub> is maximal early in the response but declines concomitant with delayed cell accumulation. Given that 15-epi-LxA<sub>4</sub> possesses antiinflammatory and well as proresolution properties, we suggest that 15-epi-LxA<sub>4</sub> acts as an internal braking signal tempering the severity and longevity of acute inflammatory responses in humans. In addition, this study shows that resolution is an active process in healthy individuals and that, unlike rodents, inflammatory responses in humans are at least dichotomous and resolution pathways dictate their longevity and eventual termination. Our findings exclude IL-10 and cAMP-elevating prostaglandin E<sub>2</sub> as factors that could potentially govern the progression and resolution of this cantharidin-induced acute inflammatory response. Furthermore, we did not find any association between inflammation profiles and age or ethnicity. However, we cannot exclude the involvement of other factors, including phagocytosis-associated molecules such as TGFβ1 (24), apoptosis-inducing factors (e.g., TNF-related apoptosis-inducing ligand) (25), cytokine clearance systems (16, 26), or antiinflammatory signaling pathways (e.g., NF-κB p50/p50) (27).

The criteria for grouping individuals as responders or nonresponders to aspirin arose from our work (18) and that of others (10), which showed that COX-2 acetylation by aspirin generates epi-Lxs. The rationale was that the more severe the inflammatory response the greater the resultant COX-2 expression and therefore biochemical machinery available for aspirin to make proportionally

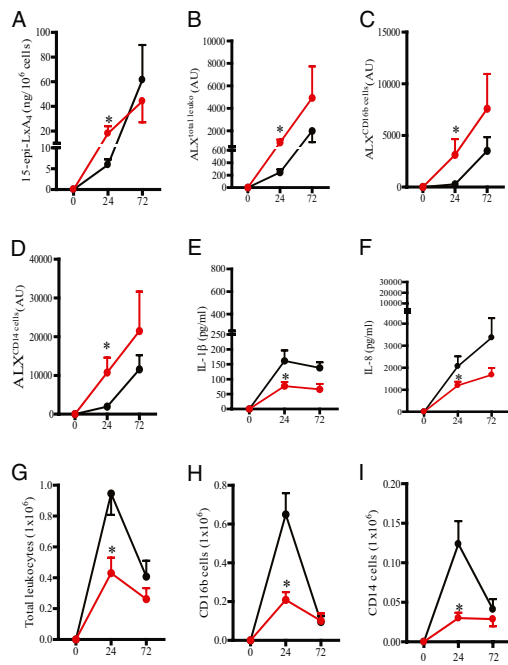


**Fig. 3.** The 15-epi-LxA<sub>4</sub> and its ALX receptor dictate inflammation severity and longevity in humans with acute inflammation. Of the endogenous anti-inflammatory and proresolution factors measured over time (in h), significant differences were found only in levels of blister fluid 15-epi-LxA<sub>4</sub> (A) as well as ALX expression on total blister leukocyte (B), CD16b-positive PMN (C), and CD14-positive monocytes/macrophages (D) between E<sup>IVS</sup> (solid line) and D<sup>IVS</sup> (dotted line). (C) Representative FACS histograms illustrate increased ALX expression on CD16b-positive PMNs in E<sup>IVS</sup> compared with D<sup>IVS</sup> with similar comparative histograms (D) illustrating increased ALX expression on CD14-positive monocytes/macrophages. Data are presented as mean ± SEM; \**P* < 0.05 and \*\*\**P* < 0.001 represent differences in inflammatory parameters between E<sup>IVS</sup> and D<sup>IVS</sup> at 24 h. AU represents arbitrary units of ALX expression intensity determined by FACS.

more antiinflammatory epi-Lxs (28). Thus, aspirin acts as a type of “magic bullet” exerting its beneficial effects in an inflammation-dependent manner. On this basis, volunteers were grouped according to those who showed a response to aspirin (following cantharidin) and those who did not. Taking this further revealed that responders to aspirin showed a resolution of inflammation more readily than those who were refractory to aspirin. As mentioned earlier, under baseline conditions (i.e., without aspirin), the key determinant of this differential inflammatory profile is 15-epi-LxA<sub>4</sub>. Importantly, although aspirin was originally shown to trigger epi-Lxs (9), we (18) and others (10) found detectable levels of 15-epi-LxA<sub>4</sub> in healthy volunteers as well as mice not given aspirin. The source of this non-aspirin-generated epi-Lx is unknown, but it is possible that COX-2 is acetylated by an endogenous acetylating agent to produce 15*R*-hydroxy eicosatetraenoic acid. Indeed, in mice dosed with COX-2 inhibitors, levels of plasma 15-epi-LxA<sub>4</sub> are significantly reduced (Fig. S2). Alternatively, epi-Lxs could be generated by a P450 pathway to produce 15*R*-hydroxy eicosatetraenoic acid (11). However, given that SKF525A [cytochrome P450 inhibitor selective for CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4/5 but not CYP1A2, CYP2A6, and CYP2E1 (29)] had no effect on plasma epi-Lxs (Fig. S2), we propose that epi-Lxs may be generated by a cell-derived acetylating agent. Therefore, from the data presented here, we hypothesize that early in the inflammatory response of E<sup>IVS</sup>, acetylation of COX-2 is low but increases as inflammation resolves, triggering increases in blister fluid epi-Lx. In contrast, in D<sup>IVS</sup>, endogenous COX-2 acetylators generate epi-Lxs

early in the response but decline in line with 15-epi-LxA<sub>4</sub> synthesis, taking the “hand brake” off inflammation. This endogenous acetylating hypothesis would certainly explain why aspirin failed to trigger 15-epi-LxA<sub>4</sub> in D<sup>IVS</sup> as the inflammatory milieu would be saturated and the addition of an exogenous acetylating agent (i.e., aspirin) would fail to further acetylate COX-2.

In our original study, ASA<sup>low</sup> did not affect blister fluid cytokine levels in 26 volunteers despite triggering 15-epi-LxA<sub>4</sub>. This was surprising given that 15-epi-LxA<sub>4</sub> inhibits cytokine and chemokine release from experimental tissue injury (30) and isolated cells stimulated in culture (31). Indeed, we found that 15-epi-LxA<sub>4</sub> inhibited a range of cytokines/chemokines from LPS-stimulated human monocytes, monocyte-derived macrophages, and human umbilical vein endothelial cells (HUVECs), with the most broad-ranging suppressive effects being on monocytes (Fig. S1). However, the presence of a group of individuals who are refractory to ASA<sup>low</sup> among those sensitive to ASA<sup>low</sup> provided the erroneous impression of an overall null effect. Separating groups based on inflammation severity/longevity revealed that those with low baseline 15-epi-LxA<sub>4</sub> and therefore high inflammation responded to ASA<sup>low</sup> because it triggered 15-epi-LxA<sub>4</sub>/ALX and therefore dampened cytokines/chemokines and leukocyte trafficking. D<sup>IVS</sup> had low cytokine/chemokine levels as a result of maximum baseline 15-epi-LxA<sub>4</sub>/ALX. As the latter was not further elevated by ASA<sup>low</sup>, these D<sup>IVS</sup> were ASA<sup>low</sup>-refractory. This does not exclude the likelihood that D<sup>IVS</sup> may become responsive to ASA<sup>low</sup> when baseline 15-epi-LxA<sub>4</sub>/ALX eventually wanes and cytokine/chemokine levels increase. How-

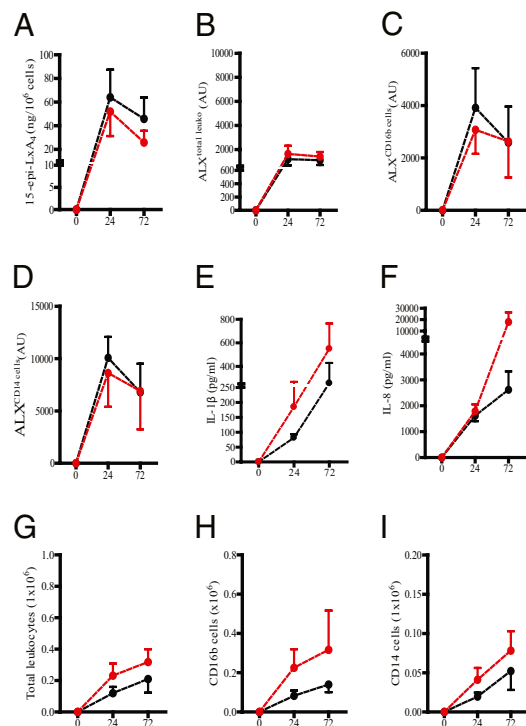


**Fig. 4.** Modulating 15-epi-LxA<sub>4</sub> with ASA<sup>low</sup> dampens inflammation in E<sup>vs</sup>. ASA<sup>low</sup> (75 mg/d for 10 d, data represented by red line) elevated 15-epi-LxA<sub>4</sub> in the blister fluids of 16 volunteers (A) as well as ALX expression on total blister leukocyte (B), PMN (C), and monocytes (D) compared with controls (black line). This elevation in endogenous 15-epi-LxA<sub>4</sub>/ALX was associated with reduced blister fluid IL-β (E) and IL-8 (F) as well as blister total leukocyte (G), PMN (H), and monocyte numbers (I). Data are presented as mean ± SEM; \*P < 0.05 represent differences after 24 h in cantharidin-induced skin blister inflammatory parameters in E<sup>vs</sup> before and after aspirin. AU represents arbitrary units of ALX expression intensity determined by FACS.

ever, we did not examine the long-term fate of inflammation/resolution in D<sup>vs</sup> because of the inherent instability of their blisters. We were also unaware of how long inflammation lasts in these responders and what proresolution pathways are required to ultimately dampen their response.

This is one of the first reports showing that modulating a proresolution pathway dampens innate immune-mediated responses in healthy humans. These individuals did not take fish oil, nor did they have their diet supplemented with docosahexaenoic acid, a precursor that, when metabolized instead of arachidonic acid down the LOX/COX pathway in the presence of aspirin, results in the generation of lipids of the resolvins D series, which dampen PMN trafficking into and clear macrophages out of sites of inflammation (32). Given the broad antiinflammatory, proresolution, and antibacterial properties of resolvins (33), it would be important to examine the effects of fish oils in general, or docosahexaenoic acid in particular, with aspirin for their combined antiinflammatory, antibacterial, and cardioprotective effects in man. That notwithstanding, ASA<sup>low</sup> has been associated with gastric bleeding, and its usefulness as a cardioprotective agent balanced against gastrointestinal side effects is of ongoing concern (34). Therefore, next generation epi-Lxs, which protect the gastric mucosa from injury (35), as well as resolvins, may replace parental aspirin as novel antiinflammatory/cardioprotective agents with reduced side effects.

In summary, we have shown that inflammation progresses at a different rate and with different degrees of severity in human skin blisters arising from differentially expressed proresolution pathways. And although this could be result from different secretion rates of proinflammatory signals (e.g., cytokines/chemokines, cell adhesion molecules), we propose that, as inflammation is tightly regulated to prevent it becoming “overexuberant,” the control of inflammation is tempered



**Fig. 5.** ASA<sup>low</sup> is without effect on D<sup>vs</sup>. ASA<sup>low</sup> (75 mg/d for 10 d; red dotted line) did not alter blister 15-epi-LxA<sub>4</sub> or ALX expression (A) on total blister leukocyte (B), PMN (C), and monocytes (D) compared with controls (black dotted line) in 10 human volunteers with a delayed resolving phenotype. Also, there was no effect of ASA<sup>low</sup> on blister fluid IL-β (E) and IL-8 (F) as well as blister total leukocyte (G), PMN (H), and monocyte numbers (I). Data are presented as mean ± SEM; \*P < 0.05. AU represents arbitrary units of ALX expression intensity determined by FACS.

by endogenous checkpoint control systems including epi-Lxs. This system is differentially expressed in different populations, resulting in different degrees of inflammation severity and longevity. Arguably, this could impact the rate at which different populations neutralize bacterial infection, for instance, or the propensity to develop chronic inflammation/autoimmunity arising from a more sustained response. Indeed, there is an inverse correlation between levels of endogenous lipoxins and disease severity in severe asthma (36), Henoch–Schonlein purpura (37), and scleroderma lung disease (38). Our findings may explain, in general, why some individuals are refractory to conventional antiinflammatory agents and importantly emphasizes the need to tailor antiinflammatory treatment regimes to individuals depending on their rate/profile of inflammatory response. These data also highlight that, when developing antiinflammatory drugs, we need to be mindful of their potentially resolution-toxic properties, i.e., their ability to subvert the body’s attempts to switch off inflammation.

### Materials and Methods

**Inflammatory Models and Drug Treatment.** Two blisters were elicited on the ventral aspect of the forearms of male healthy volunteers (aged 25–50 y) as previously described (39–42) by applying 10 μL of 0.1% Cantharone (Dormer Labs). Volunteers were of diverse ethnic backgrounds and were not taking NSAIDs or aspirin-containing medications for 2 weeks before commencement of the study. ASA<sup>low</sup> (75 mg) was taken daily for 10 d before a second set of blisters was elicited on the contralateral forearm, with aspirin consumed for the duration of the response up to 72 h. Ethical approval was obtained from UCL Ethics (project identification no. 1309/001). Blister cell numbers were enumerated by hemocytometer. For plasma lipid measurements, animals were bred under standard conditions and maintained in a 12-h/12-h light/dark

cycle at  $22 \pm 1$  °C and given food and tap water ad libitum in accordance with United Kingdom Home Office regulations.

**FACS and Lipid Mediators.** Anti-CD14, CD16b, and ALX (human) as well as isotype controls were from Serotec or BD Biosciences. Peripheral blood and blister-derived leukocytes were acquired by FACSCalibur (BD Biosciences) using appropriate compensation when necessary, and data were analyzed by CellQuest Pro. Thromboxane A<sub>2</sub> in the form of stable thromboxane B<sub>2</sub> was measured by enzyme immunoassay (GE Healthcare) and 15-epi-LxA<sub>4</sub> was measured by ELISA (Neogen). Cytokines and chemokines were measured by Multiplex cytokine array analysis (Bio-Rad) using the manufacturer's protocols.

**Leukocyte Isolation, in Vitro Cell Culturing, and Treatment Protocols.** Blood (80 mL) was collected in heparin from healthy male volunteers followed by red blood cell sedimentation using EloHAES. Remaining leukocyte-rich plasma was subjected to Percoll density gradient centrifugation to separate monocytes from platelets, PMNs, and lymphocytes. Monocyte fractions ( $1 \times 10^6$  cells) were incubated overnight and washed to remove nonadherent leukocytes. For differentiation into macrophages, monocytes were cultured in the presence of 2 ng/mL GM-CSF, which was replaced every 4 d for 12 d.

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Monocytes and macrophages were treated with 15-epi-LxA<sub>4</sub> (0.003 μg/mL) in RPMI 1640 without/without LPS (1 μg/mL, 1 h before stimulation). HUVECs (passage 1; Promocell) prestimulated with LPS (1 μg/mL) for 1 h were treated with 0.3 μg/mL 15-epi-LxA<sub>4</sub> (Calbiochem) for a further 24 h.

**Statistical Analysis.** Data were analyzed using the two-tailed paired Student *t* test for normally distributed data, with  $P < 0.05$  being considered significant. All analyses were paired Student *t* tests. Wilcoxon matched-pairs test was used to analyze data standardized to the number of cells accumulated within the blister (15-epi-LxA<sub>4</sub>/10<sup>6</sup> cells); because it did not follow a Gaussian distribution,  $P < 0.05$  was considered significant. When comparing two groups with unequal variances, a two-tailed unpaired *t* test with the Welch correction was used. For animal studies and in vitro assays, data were analyzed by ANOVA followed by followed by the Bonferroni *t* test.

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