A New Strategy for the Identification of Novel Molecules with Targeted Proresolution of Inflammation Properties

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A New Strategy for the Identification of Novel Molecules with Targeted Proresolution of Inflammation Properties

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As our understanding of inflammatory resolution increases, drugs that trigger proresolving pathways may become significant in treating chronic inflammatory diseases. However, anti-inflammatory drugs are traditionally tested during the first hours of onset (i.e., to dampen leukocyte and edema formation), and their ability to trigger proresolving processes has never been investigated. Moreover, there is no model available to screen for putative proresolving agents. In this study, we present a new strategy to identify therapeutic agents for their ability to switch inflammation off and restore homeostasis. Injecting 1.0 mg of zymosan i.p. causes transient inflammation characterized by polymorphonuclear neutrophil clearance and dominated by recently described resolution-phase macrophages along with an innate-type lymphocyte repopulation, the latter being a marker of tissue homeostasis. In contrast, 10 mg of zymosan elicits an aggressive response characterized by classically activated macrophages leading to systemic inflammation and impaired lymphocyte repopulation. Although this latter model eventually resolves, it nonetheless represents inflammation in the clinically relevant setting of polymorphonuclear neutrophil/classically activated macrophage dominance driving a cytokine storm. Treating such a reaction therapeutically with proresolution drugs provides quantifiable indices of resolution—polymorphonuclear neutrophil/macroage clearance, macroage phenotype switching (classically activated to resolution phase), and repopulation with resolution-phase macrophages—cardinal signs of inflammatory resolution and homeostasis in the peritoneum. As an illustration, mice bearing peritonitis induced by 10 mg of zymosan—were given ibuprofen, resolvin E1, a prostaglandin D2 receptor 1 agonist, dexamethasone, rolipram, or azithromycin, and their ability to trigger resolution and homeostasis in this new inflammatory setting was investigated. We present the first model for testing drugs with targeted proresolving properties using quantifiable parameters of inflammatory resolution and homeostasis. The Journal of Immunology, 2010, 184: 000–000.

Inflammation is a reaction of the microvasculature to infection, injury, or both characterized by the infiltration of polymorphonuclear neutrophils (PMNs) followed by monocytes, which differentiate locally into macrophages (1). The latter remove effete PMNs, eosinophils, or lymphocytes, and provided that the injurious agent is removed, inflammation will resolve (2–4). Importantly, the ultimate objective of resolution is to restore homeostasis and provide little opportunity for tissue injury or for the development of chronic inflammation, autoimmunity, or both. The onset of acute inflammation is well understood in terms of the mediators and mechanisms that drive that response. Indeed, targeting factors that cause inflammation, including PGs and cytokines, for instance, has provided the mainstay for treating chronic inflammatory diseases. However, nonsteroidal anti-inflammatory drugs, steroids, and anti–TNF-α are not without side effects, alleviating the symptoms without targeting the underlying disease (5, 6). Thus, there is a huge unmet clinical need for successfully treating chronic inflammation.

Unlike onset, the other end of the inflammatory spectrum, resolution, is less well understood (7). Nonetheless, we are increasingly identifying soluble mediators, cells, and signaling pathways that switch inflammation off (8). As a result, there is now interest in developing drugs that mimic the mode of action of endogenous proresolving factors to drive ongoing inflammation down a proresolving pathway. This would represent a novel approach for treating chronic inflammatory diseases. However, anti-inflammatory drugs are traditionally tested for efficacy during the first few hours of onset (i.e., to dampen leukocyte and edema formation), and their ability to trigger proresolving processes is never investigated. Indeed, there is currently no model to screen for proresolving agents. In which case, we present a new strategy based on our increasing understanding of resolution pathways to help identify novel pharmaceuticals for their ability to switch inflammation off and restore homeostasis.

Because inflammation resolves in the inflamed peritoneum, cyclooxygenase/lipoxygenase (LOX) lipid biosynthesis as well as efferocytosis are critical determinants of this process (2). This coupled with macrophages possessing a unique proresolving phenotype (9) as well as innate-type lymphocyte repopulation collectively leads to restoration of tissue homeostasis (10, 11). Collectively, these represent the cardinal signs of inflammatory resolution in the peritoneum. To illustrate this, we recently described the impact on inflammation of two distinct doses of zymosan to separate groups of mice (9). A low dose of 1.0 mg caused transient inflammation characterized by PMN clearance...
and dominated by resolution-phase (rM) macrophages along with innate-type lymphocyte repopulation. In contrast, 10 mg elicited more substantial inflammation characterized by classically activated (M1) macrophages as well as PMNs leading to systemic inflammation and impaired lymphocyte repopulation. Although this model eventually resolves, we believe that the latter is ideally suited to screen drugs for proresolution properties, because it represents inflammation in the clinically relevant setting of PMN and M1 macrophage dominance driving a cytokine storm. We argue that treating such a response therapeutically with a targeted proresolution agent provides easily quantifiable indices of resolution: PMN/macrophage clearance, macrophage phenotype switching down a proresolution pathway (M1 to rM macrophages), and repopulation of the inflamed peritoneum with resolution-phase lymphocytes.

In this study, we characterized PMN and macrophage kinetics in response to 10 mg of zymosan i.p. and highlight impaired lymphocyte repopulation and the predominance of M1 macrophages as markers of ongoing inflammation. To demonstrate the use of this model, mice bearing peritonitis induced by 10 mg of zymosan were given a nonsteroidal anti-inflammatory drug, proresolution lipids, a steroid, a phosphodiesterase IV inhibitor, or a macrolide. Their abilities

![Graphs showing the time course of resolving peritoneal inflammation in response to 1.0 mg of zymosan.](http://www.jimmunol.org/)

**FIGURE 1.** Time course of resolving peritoneal inflammation in response to 1.0 mg of zymosan. With isotype control Abs as well as (A) Gr1- (granulocytes) and F4/80-specific (macrophages) Abs, FACS was used to determine the subpopulations of cells that influxed into the peritoneum. Having counted (B) total cells by hemocytometer, we then calculated the profiles of (C) granulocytes (mainly PMNs) and (D) macrophages over time. Established resolution indices (inset) were used to denote maximum cell numbers present at the peak of the inflammatory response ($\Psi_{\text{MAX}}$), the time during inflammation when $\Psi_{\text{MAX}}$ occurs ($T_{\text{MAX}}$), and the time point during resolution when cell numbers are reduced by 50% of $\Psi_{\text{MAX}}$ ($T_{\text{50}}$), whereas the $R_i$ is the time taken for cells at $\Psi_{\text{MAX}}$ to reach $T_{\text{50}}$ (i.e., be reduced by 50%). FACS also was used to determine the influx of (E and F) lymphocytes into the peritoneum as inflammation resolved ($n = 6$-8 mice per group with data expressed as mean ± SEM).
to clear PMNs and macrophages, convert M1 to M2 macrophages, and trigger lymphocyte repopulation were used as markers of drugs exerting proresolutive and prohomeostatic properties. All of the drugs were dosed therapeutically in a pharmacologically relevant manner. For comparison, each drug also was investigated for its ability to dampen the early onset phase of acute inflammation. Thus, positive effects at onset are defined as anti-inflammatory, whereas beneficial effects in the model for 10 mg of zymosan given therapeutically are distinguished as proresolving. In this study, we present the first experimental model pertinent for testing drugs with putative proresolutive properties using easily quantifiable parameters of inflammatory resolution and homeostasis.

Materials and Methods
Animal maintenance and induction of peritonitis
C57BL/6J mice were bred under standard conditions and maintained in a 12 h light/dark cycle at 22°C and given food and tap water ad libitum in accordance with United Kingdom Home Office regulations. Peritonitis was induced by i.p. injection of either 1 or 10 mg type A zymosan (Sigma-Aldrich, Poole, U.K.) in 0.5 ml PBS after 15 s of sonication on ice. Inflammatory cells were retrieved at the time points described in the Results by injecting 2 ml sterile PBS with 3% sodium citrate, the latter being important for inflammatory reactions elicited by 10 mg zymosan. Cells were counted by hemocytometer, and exudates were stored at −80°C until further analysis. This study received institutional review board approval for the use of mice from the United Kingdom Home Office.

Drug administration and cell retrieval
1.0 mg zymosan. Ibuprofen (100 mg/kg), rolipram (30 mg/kg), dexamethasone (0.5 mg/kg) (all from Sigma-Aldrich), or azithromycin (30 mg/kg; Fluka Chemika-BioChemika, Buchs, Switzerland) was given orally by suspension in 1% gum tragacanth (Sigma-Aldrich) 1 h before zymosan injection. 15-Epi-lipoxin A4 (1 μg per mouse, RX-10001-23-24, a sodium salt of RvE1 provided by Resolvys Pharmaceuticals, Boston, MA) and the prostaglandin D2 receptor 1 (DP1) receptor antagonist BWA868C (10 μg per mouse; Cayman Chemical, Ann Arbor, MI) were given i.p. 5 min before zymosan injection. For all of the drugs, inflammatory exudates containing leukocytes were collected 4 h after zymosan injection.

10 mg zymosan. Drugs were administered therapeutically 72 h after zymosan injection at the above doses and routes of administration, with the exception of 15-epi-lipoxin A4, which was injected i.p., and the DP1 receptor agonist BW245C (0.3 mg/kg) was used instead of the receptor antagonist. Exudates containing inflammatory cells were collected 4 h later.

Ex vivo macrophage culture
Peritoneal washouts were treated with ACK lysis buffer (Sigma-Aldrich) to remove RBCs. After being washed, peritoneal cells were suspended in DMEM (Invitrogen, Paisley, U.K.) supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. Remaining cells (1.0 × 10^6) were seeded into 100 mm dishes in 10 ml DMEM containing 10% FBS and antibiotics. After 18 h, nonadherent cells were removed by washing with PBS. The adherent cell population was used for all experiments.

FIGURE 2. Time course of peritoneal inflammation in response to 10 mg of zymosan. With isotype control Abs as well as (A) Gr1− (granulocytes) and F4/80-specific (macrophages) Abs, FACS allowed us to determine subpopulations of cells that influxed into the inflamed peritoneum in response to 10 mg of zymosan. Having counted total cells by hemocytometer, we then calculated the profiles of each cell subpopulation (B–D) over time using established resolution indices (inset). YMAX denotes maximum cell numbers present at the peak of the inflammatory response, whereas TMAX is the time during inflammation when YMAX occurs. T50 is the time point during resolution when cell numbers are reduced by 50% of YMAX, whereas the Ri is the time taken for cells at YMAX to reach T50 (i.e., be reduced by 50%). E. Lymphocytes repopulated the cavity from day 6, once inflammation in the peritoneum resolved (n = 6–8 mice per group with data expressed as mean ± SEM).
in a 24-well plate and left to adhere for 1 h in a humidified CO₂ incubator. After three successive washes with PBS, cells were trypsinized and contaminating B lymphocytes were removed using magnetic CD19 beads (Miltenyi Biotec, Auburn, CA). Purified macrophages then were incubated at 250 x 10⁶ cells per well in 0.5 ml DMEM. After 6 h, cell-free supernatants were removed and stored at -280°C for macrophage phenotypic analysis.

Cytokine and FACS analysis

Concentrations of TNF-α (eBioscience, San Diego, CA) and IL-10 (BD Pharmingen, San Diego, CA) were determined in the exudates and macrophage supernatants by ELISA according to the manufacturer’s instructions. FACS was performed by using FACScanB (BD Biosciences, San Jose, CA) with data analysis by CellQuest software. Leukocytes were incubated with Abs to Gr1 (Ly6C/LY6G), Ly6C (BD Pharmingen), F4/80 containing Fc blocker (eBioscience), or CD3 (AbD Serotec, Raleigh, NC) using isotype-specific Abs from each respective supplier and with compensation where appropriate for dual labeling prior to the FACS analysis.

Statistical analysis

The two-tailed Student t test or ANOVA was used to analyze data. A value of p < 0.05 was deemed statistically significant. Data are presented as mean ± SEM.

Results

Inflammatory cell trafficking in response to 1.0 mg zymosan

To provide a comparison with the more aggressive inflammation elicited by 10 mg of zymosan, we first characterized the inflammatory
response in animals injected with a low dose (1.0 mg) of zymosan by FACS. This triggered an immediate influx of Gr1-positive granulocytes (the majority being PMNs) peaking at 4 h and waning by 48-72 h with F4/80-positive macrophages appearing at ~24 h and persisting up to resolution (Fig. 1A). To quantify this in a temporal fashion, we used recently described parameters for leukocyte trafficking into and out of the inflamed peritoneum to quantify cell trafficking (12). For example, $\Psi_{\text{MAX}}$ denotes maximum cell numbers present at the peak of the inflammatory response, whereas $T_{\text{MAX}}$ is the time during inflammation when $\Psi_{\text{MAX}}$ occurs. $T_{\text{50}}$ is the time point during resolution when cell numbers are reduced by 50% of $\Psi_{\text{MAX}}$, whereas the resolution interval (RI) is the time taken for cells at $\Psi_{\text{MAX}}$ to reach $T_{\text{50}}$ (i.e., be reduced by 50%). Thus, 1.0 mg of zymosan resulted in $12 \times 10^6$ ($\Psi_{\text{MAX}}$) total inflammatory leukocytes present in the peritoneum at the height of the reaction ($T_{\text{MAX}} = 24$ h), being reduced by 50% at 48 h resulting in the RI of 24 h (Fig. 1B). PMNs totaled $8.4 \times 10^6$ ($\Psi_{\text{MAX}}$) at 4 h ($T_{\text{MAX}}$), taking 44 h (RI) to clear by 50% (Fig. 1C). Macrophages followed a profile similar to that of total cells but with a $\Psi_{\text{MAX}}$ of $5.9 \times 10^6$ (Fig. 1D). The rate of total cell influx in the first 4 h was $2.8 \times 10^6$ cells per hour, which decreased to $3.7 \times 10^5$ cells per hour between 4 and 24 h. Thereafter, cells vacated the cavity, leading to resolution. Of the total cells, PMNs were the principle cell that trafficked in response to zymosan, doing so at a rate of $2.1 \times 10^5$ cells per hour within the first 4 h, whereas macrophage trafficking followed that of PMNs at a rate of $73 \times 10^3$ cells per hour within the first 4 h, increasing to $281 \times 10^3$ cells per hour from 4 to 24 h. From 24 h, macrophages cleared to the parathymic lymph nodes (13).

Another parameter of resolution is lymphocyte influx (10, 11). Postresolution peritoneal lymphocytes have no role in switching inflammation off (with the likely exception of NK cells) but are essential for controlling the severity of subsequent inflammatory responses. Thus, CD3 and CD19 influx occurs once inflammation abates, which we contend constitutes the restoration of tissue homeostasis. In animals injected with 1.0 mg of zymosan, CD3 and CD19 cells repopulated the cavity from 12 h onward, peaking in numbers as inflammatory leukocytes vacate the cavity (Fig. 1E, 1F).

**FIGURE 4.** Schematic of the pro-resolution drug screening strategy. A, Sequence of events that occurs in response to 10 mg of zymosan injection, characterized by large inflammatory cell infiltrate, M1 macrophage phenotype, and impaired lymphocyte repopulation, which in the peritoneum are the cardinal signs of resolution and restored homeostasis depicted in B in response to 1.0 mg of zymosan. The objective of a drug that elicits pro-resolving properties when given therapeutically at the height of inflammation is to drive that response down a proresolving pathway.

**B**

**Resolving inflammation**

- 0.1 mg zymosan
  - M1 phenotype switching
  - PMN clearance
  - Lymphocyte repopulation
  - Resistance to pro-inflammatory cytokine generation

**A**

**Inflammation**

- 10 mg zymosan
  - M1 macrophages
  - Impaired lymphocyte re-population
  - Pro-inflammatory cytokines

**Naive**

- Onset
- Inflammatory
- Resolution/homeostasis

- Lymphocytes

- (M1 macrophages)
FIGURE 5. A–G, Effects of various classes of anti-inflammatory drugs for their proresolution properties: leukocyte clearance. Members of established therapeutics as well as novel proresolution lipids were chosen to determine their ability to push ongoing inflammation down a proresolution pathway as a means of emphasizing the utility of this model. All of the drugs were administered at 72 h to mice bearing peritonitis induced by 10 mg of zymosan because not only is it the peak of inflammation but it is also the time after which there is active clearance of inflammatory cells. Therefore, any effect seen will arise from enhanced clearance (resolution) rather than anti-inflammation (dampened cell influx) ($n = 6–8$ mice per group with data expressed as mean ± SEM).
Inflammatory cell trafficking in response to 10 mg zymosan

For animals injected with 10 mg of zymosan, inflammation was monitored over 9 d due to the increased severity of the stimulus (9). FACS depicted the temporal change in Gr1- and F4/80-positive granulocytes and macrophages, respectively, throughout the response (Fig. 2A). Total cell numbers reached $\sim 27 \times 10^6$ (T\text{MAX} = 72 h) at peak, taking 24 h to clear by 50%, with full resolution seen at 216 h (Fig. 2B). T\text{MAX} for PMNs was $7 \times 10^6$ at 48 h, requiring a further 48 h to clear (Fig. 2C), whereas macrophages were the dominant cell type, with a T\text{MAX} of $12 \times 10^6$ at 72 h, remaining in the cavity for a further 72 h (R\text{C} max) (Fig. 2D). Lymphocytes only began to appear in this model 6 d after zymosan injection when PMNs and macrophages cleared the inflamed peritoneum. The rate of total cell influx in the first 24 h was $597 \times 10^3$ cells per hour, decreasing to $\sim 379 \times 10^3$ cells per hour from 24 to 48 h and down to $\sim 120 \times 10^3$ cells per hour between 48 and 72 h (Fig. 2E). As with a low dose of zymosan, PMN influx was rapid, being $338 \times 10^3$ within the first 24 h, slowing to $\sim 10^3$ between 24 and 48 h, whereas macrophage influx was delayed, starting at 24 h with a rate of $245 \times 10^3$ cells per hour up to 48 h and $34 \times 10^3$ cells per hour between 48 and 72 h.

Cytokine response in inflammation induced by 1.0 and 10 mg zymosan

Levels of inflammatory cytokines were measured in the exudates of animals injected with either 1.0 or 10 mg of zymosan at 72 h. Most cytokines were low in resolving exudates, being expectedly higher in those of animals injected with 10 mg of zymosan (Fig. 3A-I). Moreover, the ability to trigger cytokines above baseline with LPS (i.p. into animals bearing 1.0 mg of zymosan at 72 h) during resolution was impaired, whereas injection of LPS into the peritoneum of mice bearing 10 mg of zymosan at the same time point resulted in elevated cytokine synthesis. We interpret these data as resolution being an immune-suppressed state where responses to subsequent inflammatory stimuli are dampened, an effect not seen in animals bearing an aggressive inflammation and populated with M1 macrophages. We also compared the macrophages from mice injected with 10 mg of zymosan to those from the readily resolving model, finding, as shown previously (9), that resolving macrophages synthesize more IL-10 than M1 macrophages (Fig. 3J).

Proresolution drug screening strategy

The overall objective of this study was to develop a model of inflammation that will be useful in determining whether a novel molecule has proresolution actions. To this end, we propose that such molecules, when injected therapeutically at 72 h in murine peritonitis triggered by 10 mg of zymosan (Fig. 4A), will drive that response down a proresolution pathway, as depicted by events shown in Fig. 4B. Using this simple strategy, to validate the model we screened a series of molecules with known anti-inflammatory properties for their ability to trigger resolution.

Proresolution potentials of diverse classes of anti-inflammatory agents

All of the drugs were administered at 72 h to mice bearing peritonitis induced by 10 mg of zymosan because not only is it the peak of inflammation but it is also the time after which there is active clearance of inflammatory cells. Therefore, any effect seen will arise from enhanced clearance (resolution) rather than anti-inflammation (dampened cell influx). In addition, the critical proresolution parameters of M1 to rM macrophage switching as well as lymphocyte repopulation also were determined. Dexamethasone, azithromycin, 15-epi-lipoxin A$_4$, and RvE1 exerted the most consistent proresolving effects, bringing about enhanced clearance of total cells, PMNs, and macrophages (Fig. 5). Rolipram tended toward enhanced monocyte and macrophage clearance, being only significant with PMNs, whereas ibuprofen and a DP1 receptor agonist caused a significant clearance in PMNs only. Ibuprofen and RvE1 elevated CD3-positive lymphocytes, with all of the other nonsteroidal anti-inflammatory drugs having no effect (15-epi-lipoxin A$_4$ and rolipram) or inhibiting (dexamethasone, azithromycin, and DP1) their repopulation. Macrophages in the peritoneum of mice injected with 10 mg of zymosan possess an M1 macrophage phenotype (9), generating less IL-10 and more TNF-$\alpha$, for instance, than rM macrophages. Only RvE1 elevated IL-10 synthesis from M1 macrophages when incubated ex vivo, suggesting a potential change toward a rM macrophage phenotype (Fig. 6).

Thus, we have used a model of peritonitis characterized by aggressive inflammation and lacking any soluble mediator or cellular marker of resolution with which to screen existing anti-inflammatory molecules for their proresolution abilities. We therefore are proposing, for the first time, a strategy with which to screen drugs with potential proresolution properties. Of the molecules tested in this report, none exerted all of the required pro-resolution properties, with perhaps the exception of RvE1 and 15-epi-lipoxin A$_4$, with RvE1, in particular, dampening total cells, triggering CD3 repopulation, and potentially reverting M1 cells toward a rM macrophage phenotype.

Anti-inflammatory molecules on the onset of inflammation

For comparison, all of the above molecules were screened to determine their effects on the traditional early phase of acute inflammation. To this end, drugs were administered 30 min to 1 h prior to injection of 1.0 mg of zymosan, and their impact on inflammation was determined 4 h later (Fig. 6). Total cells, PMNs, and monocytes were counted while macrophages were too few at this phase. As can be seen from Fig. 7, all of the drugs with the exception of RvE1 altered inflammatory cell trafficking into the inflamed cavity. Levels...
of cytokines were either inhibited or not altered in response to all of the test drugs with the exception of differential effects of ibuprofen, which triggered TNF-α and dampened IL-10 (Fig. 8), despite reducing total inflammatory cell influx.

**Discussion**

In response to a mild stimulus (1.0 mg of zymosan), peritoneal inflammation normally resolves between 24 and 72 h and is characterized by cytokine and chemokine clearance (14), a transition in populations of PMNs to predominantly macrophages (15), apoptosis and phagocytosis (16), cyclooxygenase/LOX-derived lipid biosynthesis (17), rM macrophages (9), and repopulation of the resolving peritoneum with innate-type as well as CD3/CD19 positive lymphocytes (10, 11). The latter hails the final stages of resolution and restoration of tissue homeostasis in the murine peritoneum. We investigated the absence or dysregulation of some of these pathways during the same time frame in animals injected with 10 mg of zymosan. There was a predominance of PMNs and M1 macrophages with few repopulating innate-type lymphocytes. Although this model eventually resolves from 6 to 9

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**FIGURE 7.** A–G. All of the drugs screened for putative proresolving properties also were administered to mice at the early onset phase of acute inflammation, the phase typically used to determine drug anti-inflammatory efficacy (i.e., 0.5 or 1 h before injection of 1.0 mg of zymosan). Their effects on total leukocyte numbers as well as PMNs and monocytes were determined 4 h later (n = 6–8 mice per group with data expressed as mean ± SEM).


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A

B

C

D

E

F

G

FIGURE 8. A–G. All of the drugs screened for putative proresolution properties also were administered to mice at the early onset phase of the response. This phase traditionally was used to determine drug anti-inflammatory properties (i.e., 0.5 or 1 h before injection of 1.0 mg of zymosan), and their effects on cell-free exudate cytokine levels were determined 4 h later (n = 6–8 mice per group with data expressed as mean ± SEM).

d, the phase encompassing 24–72 h is arguably similar to ongoing inflammation associated with, for instance, chronic obstructive pulmonary disease, chronic granulomatous disease, or sepsis. Without a doubt, the exact nature of the inflammation elicited by 10 mg of zymosan is not strictly identical to inflammation that drives the above diseases. Nonetheless, it represents inflammation in its ongoing state and offers an opportunity to determine the efficacy of drugs administered in a clinically relevant setting (i.e., when the disease is established and ongoing and not prophylactically before inflammation has started). Moreover, it will distinguish between molecules that are anti-inflammatory (dampen cell influx) and those that trigger resolution and restore normal tissue function. We argue that this model represents the first step in testing the efficacy of novel therapeutics with targeted proresolution properties.

Given the short half-life in rodents of the molecules used in this report (4–7 h), we did not dose for the duration of the Ri (24–48 h depending on cell types) in inflammation triggered by 10 mg of zymosan but opted for a single administration at 72 h, examining their effects on total cells, PMNs, and macrophage numbers 4 h later. In our experience, repeated dosing into the peritoneum can affect inflammation and therefore was avoided (unpublished observations). Nonetheless, some researchers may find it more appropriate to test for shortening of the Ri for drugs administered i.v., orally, or s.c. In terms of the stimulus used, we used sonicated zymosan, because in our experience it is robust and provides highly reproducible results within a given batch provided it has been prepared as previously described (18, 19) and stored at −80˚C. However, batch-to-batch variation can be substantial, and preliminary experiments must be carried out to ensure that the levels of zymosan used give the outcome desired.

Besides inflammation triggered by 10 mg of zymosan, molecules could be administered for self-limiting inflammatory responses (e.g., those triggered by 1.0 mg of zymosan) just prior to resolution, with a shortening of the Ri used to determine proresolution efficacy (12). This is also a reasonable approach and one that may be considered by some investigators. Indeed, it would also give some insight into molecules that exert so-called resolution-toxic effects (3), because molecules that unwittingly impair resolution may be highlighted more readily than if the model with 10 mg of zymosan is used. Nonetheless, we opted for the latter, because it is more clinically relevant and novel agents that bring about resolution here would arguably do so in inflammation triggered in response to a low dose of zymosan. However, the converse may not always hold true.

In this study, we screened a series of established anti-inflammatory drugs as well as molecules currently the focus of interest in the scientific literature. There was no single agent that triggered all of the cardinal signs of resolution, although RvE1 and 15-epi-lipoxin A4 followed by dexamethasone showed the most resolution-positive properties. All of the other molecules that were investigated affected disparate aspects of the resolving process. Results obtained with RvE1 and 15-epi-lipoxin A4 are not surprising given their well-established proresolution properties (17). However, the effects seen with dexamethasone and azithromycin are of interest, because they suggest that these molecules clear PMNs as well as macrophages from the inflamed peritoneum in an active manner. Although dexamethasone is known to enhance macrophage phagocytosis of apoptotic PMNs (20), this is only after 24 h of pretreatment in vitro. It is therefore unlikely that dexamethasone’s enhanced clearance of PMNs found here is via efferocytosis, because its effects were examined just 4 h after administration, suggesting a nongenomic mechanism of action. Macrolides have been suggested to exert immune-regulatory and proresolving properties (21) but had a negative effect on CD3 cells in the peritoneum. Nonetheless, understanding the likely signaling mechanism by which dexamethasone and azithromycin cause such rapid PMN and macrophage clearance will provide valuable new insight into hitherto unknown proresolution pathways.

The definition of resolution presented here (cyclooxygenase/LOX lipid biosynthesis, efferocytosis, rM macrophage occupation, and innate-type lymphocyte repopulation) pertains to the peritoneal cavity. And although some of these processes may be conserved, it is likely that not all will occur with equivalent importance in all tissues. Moreover, proresolution pathways may also be stimulus specific. For example, there is a greater importance for γδ T cells in
the resolution of infectious inflammation (22) than there is for these innate-type lymphocytes in resolving sterile inflammation (11). Certainly, there seems to be little current evidence that innate-type and CD3/CD19 positive lymphocytes repopulate lungs of human recovery from Streptococcus pneumoniae (C. Haslett, personal communications), although this has yet to be verified experimentally. In which case, when developing targeted proresolving drugs, these may have to be tailored to the organ, disease, and initiating stimulus.

In summary, we suggest that molecules exerting proresolution properties should be developed with the overall philosophy of driving ongoing and chronic inflammatory disease down a proresolution pathway. To facilitate this, we propose the use of a mouse injected i.p. with 10 mg of zymosan, because this model is aggressive in nature and at 72 h is apparently devoid of key proresolving pathways. Thus, any molecule that restores resolution events typical of this model and switches off the response possesses bona fide resolution properties in contrast to being anti-inflammatory—dampening edema and leukocyte influx. Perhaps one additional determinant of true resolution not tested in this report is to wait for the half-life of the molecule to elapse and determine any potential rebound effects. This would distinguish between transient and permanent resolution. In addition, hidden among the current pharmacopeia, may lay drugs with hitherto unappreciated proresolving action, which may provide additional insight into to the etiology of complex diseases.

Disclosures

D.W.G. is a consultant for Resolvyx Pharmaceuticals. This company is interested in developing resolvin analogs for the treatment of inflammatory diseases. Specific to this declaration is the inclusion of one such compound, RvE1, in Figs. 5–8.

References