

Nonresolving Inflammation

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Nonresolving inflammation is a major driver of disease. Perpetuation of inflammation is an inherent risk because inflammation can damage tissue and necrosis can provoke inflammation. Nonetheless, multiple mechanisms normally ensure resolution. Cells like macrophages switch phenotypes, secreted molecules like reactive oxygen intermediates switch impact from pro- to anti-inflammatory, and additional mediators of resolution arise, including proteins, lipids, and gasses. Aside from persistence of initiating stimuli, nonresolution may result from deficiencies in these mechanisms when an inflammatory response begins either excessively or subnormally. This greatly complicates the development of anti-inflammatory therapies. The problem calls for conceptual, organizational, and statistical innovations.

Inflammation is a frequent occurrence because we are partly microbial and we move in a microbial world. These circumstances ensure countless encounters with microbial stimuli in the context of tissue injury. The conjunction of these two types of stimuli in time and space is what most often initiates inflammation (Nathan, 2002). The usual result of inflammation is protection from the spread of infection, followed by resolution—the restoration of affected tissues to their normal structural and functional state.

The problem with inflammation is not how often it starts, but how often it fails to subside. Perhaps no single phenomenon contributes more to the medical burden in industrialized societies than nonresolving inflammation. Nonresolving inflammation is not a primary cause of atherosclerosis, obesity, cancer, chronic obstructive pulmonary disease, asthma, inflammatory bowel disease, neurodegenerative disease, multiple sclerosis, or rheumatoid arthritis, but it contributes significantly to their pathogenesis.

This illustrative but noncomprehensive review begins by describing different forms of nonresolving inflammation. We then discuss damage of uninfected tissues as a propagator of inflammation. This is a critical conceptual issue, because microbial stimuli are not implicated in many forms of chronic inflammation, and it is instructive to appreciate the adaptive value of our ability to launch a tissue-damaging process triggered by tissue damage itself. Next, we ask two questions interchangeably that mirror each other: How does inflammation resolve? How can resolution fail? Given that the answers are complex and incomplete, we close by considering implications for the future of anti-inflammatory therapy.

Examples of Nonresolving Inflammation

Nonresolving inflammation can take distinct histologic forms, as illustrated in Figure 1. These can succeed each other or coexist in different sites in an affected organ.

Inflammation sometimes progresses from acute to chronic and then stalls for a prolonged period, although signs of acute inflammation, such as accumulation of neutrophils, may reappear later. Classic examples involve persistent infections. The contribution of inflammation to pathogenesis deserves emphasis when the host inflammatory response, not toxins from the pathogen, is chiefly responsible for the damage to the host. Globally, tuberculosis is probably the most prevalent example. Chronic inflammation surrounding *Mycobacterium tuberculosis* can persist for decades. When the inflammation is extensive enough, it liquefies lung. The host suffers loss of respiratory capacity and sometimes blood; the pathogen gets a ride on infectious droplets to enter another host.

An enormous proportion of the global burden of disease involves nonresolving inflammation that appears to be chronic from the outset, in that the first cellular signs of inflammation involve infiltration of the tissue by monocytes, dendritic cells, and macrophages. Examples include atherosclerosis (Galkina and Ley, 2009), obesity (Nathan, 2008), and some cancers (Mantovani et al., 2008).

Frequently, acute and chronic inflammation coexist over long periods, implying continual reinitiation. Examples are found in rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, multiple sclerosis, Crohn's disease, ulcerative colitis, and cancers whose stroma is infiltrated both by macrophages and immature myeloid cells (Mantovani et al., 2008). For example, in rheumatoid arthritis, the synovium presents a striking picture of chronic inflammation, with extensive infiltration by macrophages and lymphocytes and activation of synoviocytes. In contrast, the synovial fluid is a sea of neutrophils. The effusion in an affected joint of an untreated patient with rheumatoid arthritis can be invaded by over a billion neutrophils per day that have a half-life of about 4 hr (Hollingsworth et al., 1967). Neutrophils contain cytosolic peptidyl arginine deiminase type 4, an enzyme whose activity depends on the levels of Ca²⁺ found

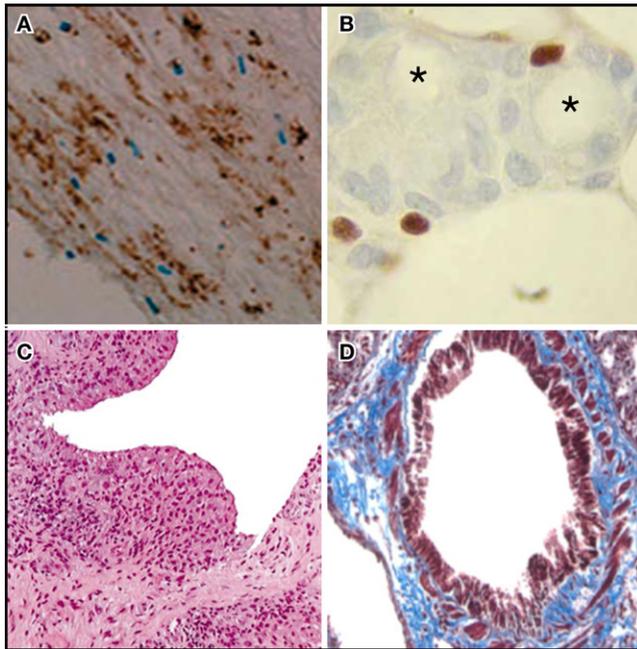


Figure 1. Examples of Nonresolving Inflammation

(A) Atherosclerosis. Plaque from a human carotid artery containing macrophages stained with anti-CD68.

(B) Obesity. Abdominal adipose tissue from an obese mouse with necrotic adipocytes (asterisks) surrounded by macrophages. Nuclei of some T cells are stained with anti-Foxp3.

(C) Rheumatoid arthritis. Synovium from a human knee infiltrated by lymphocytes, monocytes, and activated fibroblasts. Cells in the joint space (top), mostly neutrophils, are lost in sample preparation.

(D) Pulmonary fibrosis after conditional overexpression of TGF- β . Collagen is stained blue; smooth muscle cells are stained brown.

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in extracellular fluid. When neutrophils die, this enzyme may be released and activated. It may then convert the guanidino side chains of L-arginine residues to ureido residues, generating citrulline in some proteins in the joint. The autoantibodies most closely associated with the pathogenesis of rheumatoid arthritis react with citrullinated proteins (Uysal et al., 2009). Thus, dying neutrophils may help sustain an ongoing antigen-antibody reaction that attracts and activates more neutrophils, whose secretion of oxidants and proteases is synergistically destructive (Han et al., 2005).

Successful postinflammatory tissue repair requires the coordinated restitution of different cell types and structures, not only

epithelial and mesenchymal cells but also extracellular matrix and vasculature. Chemokines are critical to vascular remodeling after inflammation (Strieter et al., 2007). Without appropriate restitution of vasculature, altered tissue oxygenation may preclude normal repair, resulting in atrophy or fibrosis. When these processes destroy an organ, we do not know how to restore normal function short of replacing the organ by transplantation. Atrophy refers to the loss of parenchymal cells. For example, atrophy of the stomach follows longstanding inflammation caused by infection with *Helicobacter pylori*, resulting in loss of mucosal function and failure to produce gastric acid. Atrophy is often accompanied by expansion of extracellular tissue elements, particularly collagen, resulting in fibrosis, the deposition of excess connective tissue. It is likely that atrophy sometimes promotes fibrosis, fibrosis sometimes promotes atrophy, and each can occur independently. Both can arise without known preceding inflammation.

Fibrosis sufficient to interfere with organ function is a major medical problem after inflammation of arteries caused by accumulation of cholesterol, inflammation of the liver caused by viruses, alcohol, toxins or schistosome infections, inflammation of the lung associated with asthma or radiotherapy, and inflammation of the bowel in Crohn's disease, where fibrotic strictures (occlusions) often require surgery. Fibrosis arises from the excessive number, activity, or life span of collagen producing cells—activated fibroblasts, epithelial cells that undergo transformation to a mesenchymal phenotype, hepatic stellate cells that generate myofibroblasts, and bone marrow-derived fibrocytes that enter an affected organ from the circulation. Various chemokines attract fibrocytes into an inflammatory site, where TGF- β promotes their differentiation (Abe et al., 2001; Strieter et al., 2007). Also important are the factors governing the apoptosis of collagen-producing cells during the resolution of inflammation.

TLR agonists can activate fibroblasts directly (Wynn, 2008). Cytokines with a prominent role in promoting fibrosis include TGF- β , IL-13, IL-4, IL-6, and IL-21. Directly or through their influence on chemokine expression, these cytokines can recruit and augment the proliferation of fibrocytes, fibroblasts, and myofibroblasts and promote their production of collagen (Wilson and Wynn, 2009; Wynn, 2008). IL-4 induces macrophages to express TGF- β , PDGF, and arginase. Ornithine, a product of arginase, is a significant source of the proline and hydroxyproline that together account for almost a quarter of the residues in collagen. TGF- β activity is post-translationally regulated by release from latency-associated protein, often by proteolysis. For this and additional reasons, the balance between proteases and antiproteases (which in turn is critically regulated by reactive oxygen intermediates, or ROI) may play a large role in the degree of fibrosis that follows inflammation. TGF- β induces mesenchymal cells to express NADPH oxidase 4 (NOX4), whose production of ROI mediates TGF- β -dependent myofibroblast differentiation and extracellular matrix production (Hecker et al., 2009). IL-13 promotes both the production of TGF- β by macrophages and its proteolytic activation, although IL-13 can also promote fibrosis independently of the TGF- β /Smad signaling pathway (Wynn, 2008). Macrophage- and fibroblast-derived angiotensin II is another profibrotic stimulus that works

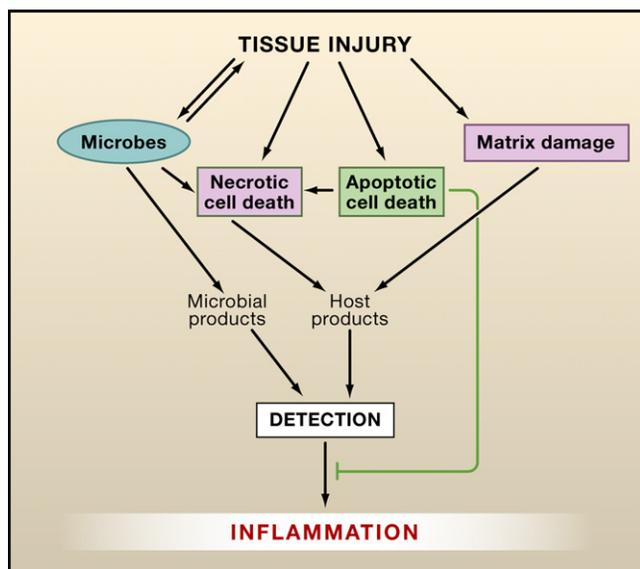


Figure 2. Initiation of Prolonged Inflammation Usually Requires Signals from Both Microbes and Injured Tissue

Transient, functionally consequential inflammation can also arise when large numbers of host cells undergo necrotic death without involvement of microbial products, most often from ischemic events. Their products activate many of the receptors that detect microbes.

at least in part through augmentation of TGF- β production (Wynn, 2008). Bioactivity of TGF- β is further controlled by the expression of matrix proteins that bind it in an inactive state. Endogenous antagonists of fibrosis include IFN- γ , IL-12, IL-10, and IL-13 α R2, a decoy receptor (Wynn, 2008). Shared actions of IFN- γ and IL-10 are uncommon, and it will be fruitful to learn more about how each of them suppresses fibrosis.

In sum, tissue healing has features in common with tissue development, which requires involution of pre-existing tissue elements. However, distinct challenges arise when the pre-existing tissue has been damaged and inflamed. It is not just the expression or extinction of certain mediators that is critical, but the orchestration of their succession—that is, their tuning and timing.

Tissue Damage as an Initiator or Propagator

As hinted above, inflammation can reduce any site in the body to a liquid that contains few living cells or intact macromolecules, or harden it into a mass packed with infiltrating lymphohematopoietic cells or stiffened by collagen. What if tissue damage, in turn, were to trigger inflammation? Wouldn't that set up a positive feedback loop that could send us all to an early grave?

By itself, sterile tissue injury, as generated by well-performed surgery, provokes little or no clinically apparent inflammation. Fortunately, since we are human-microbial consortia, the presence of microbial products in the absence of tissue injury is noninflammatory as well. As mentioned, it is when signals arising from tissue injury coincide with signals arising from microbes that inflammation usually ensues (Nathan, 2002). Nonetheless, inflammation does arise when large numbers of host cells die in place, such as in a heart attack or stroke. Although inflamma-

tion in such settings usually resolves quickly, it often contributes to the damage precipitated by the underlying event.

Why have we evolved to allow noninfectious cell death to trigger a response that exacerbates noninfectious cell death? An evolutionary perspective suggests an explanation, as summarized in Figure 2. The host senses microbial products in two ways: directly, via host molecules that bind microbial products, and indirectly, by host molecules that detect products of host cell necrosis. Microbial products can reach host detectors in two ways: by direct contamination of tissues, such as via cuts, bites or burns, or by diffusion of secreted, shed, or released microbial products through extracellular fluid, lymph, or blood. The host needs to be particularly vigilant in detecting microbial toxins that diffuse or circulate because toxins can allow a tiny bacterial biomass to incapacitate a vastly larger host. Relying on a receptor to detect a toxin may provide less warning than relying on multiple receptors to detect the damage the toxin causes. Making an advantage of necessity, the host uses necrosis of its cells as one of the immune system's earliest and best-amplified signals to report the dissemination of a microbial toxin. In evolution, sterile surgical technique was unknown, ischemic events like heart attacks and strokes—largely afflictions of the elderly—would not have imposed large selective pressures, and most cases of tissue injury that exerted large selective pressures in individuals with reproductive potential were contaminated by or caused by microbes. Thus, it is plausible that animals have evolved to interpret necrotic host cells as a sign of infection.

The molecular signals of host cell necrosis that trigger inflammation are still being defined and debated (Kono and Rock, 2008), but they are many and diverse. Examples include uric acid, adenosine, oxidized 1-palmitoyl-2-arachidonyl-phosphatidylcholine (Imai et al., 2008), chromosomal DNA (Okabe et al., 2009), IL-1 α , IL-33, high-mobility group box-1 (HMGB1), non-muscle myosin recognized by complement-fixing antibodies present in normal mice (Zhang et al., 2006), and fragments of extracellular matrix proteins generated by release of proteases from dying cells (Kono and Rock, 2008). Reflecting the extremely close relationship between detection of microbial products and detection of host cell injury, many, perhaps most, of the host molecules that specifically recognize microbial products also recognize one or another host product whose formation or relocalization reports host cell necrosis.

This sets the stage to consider mechanisms that drive the resolution of inflammation and how they may sometimes fail, as summarized in Figure 3.

Mechanisms of Resolution and Nonresolution

We avoid spontaneous inflammation through a set of tonically operating anti-inflammatory mechanisms. This was inferred from the phenotype resulting from loss-of-function mutations in what were earlier counted as over 50 genes (Nathan, 2002), and are now recognized as at least 81 (Table 1; for abbreviations and references, see Table S1 available online). These mutations lead to spontaneous emergence of persistent inflammation in people living in normal conditions or in laboratory mice during standard husbandry—that is, without a known inflammatory provocation, and without evident autoimmunity. Apparently, it

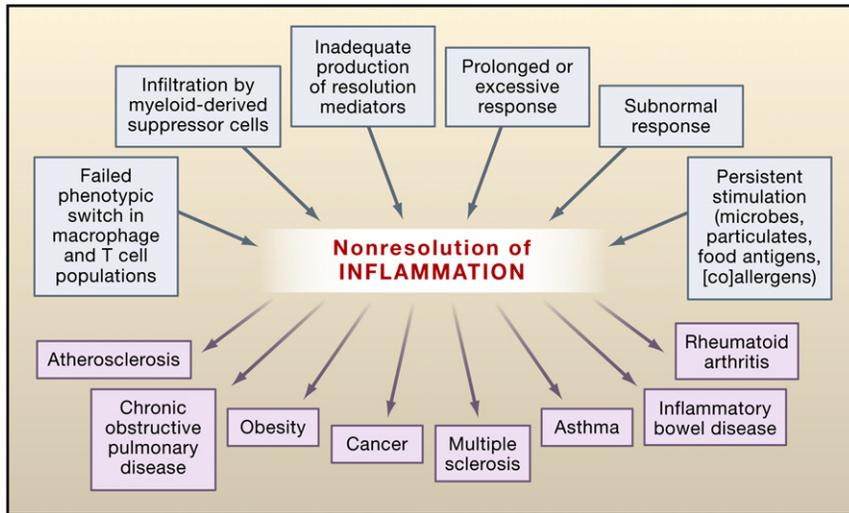


Figure 3. Mechanisms and Consequences of Nonresolution of Inflammation

developed neutrophilic accumulations in their small intestines, along with abscesses and ulcers (Kaser et al., 2008). In humans, hypomorphic variants of XBP1 are associated with Crohn’s disease (Kaser et al., 2008).

It is likely that mechanisms of similar diversity and complexity operate to resolve inflammation once it has arisen. Below, we illustrate some of the cellular and soluble factors involved. Space is lacking to discuss the many intracellular signaling molecules that impose checks and balances on proinflammatory path-

takes a complex, coordinated response by 81 or more gene products to prevent inflammation from arising spontaneously. The true number is likely to be higher, given what this list implies: that spontaneous inflammation may arise whenever we lose a nonredundant component of a mechanism that regulates proliferation or signaling in lymphoid, myeloid, or epithelial cells responding to antigens, microbes, or injury.

Examples involving those three cell types are instructive. TNF- α -induced protein-2 (TIPE2) is selectively expressed in lymphoid cells and acts to dampen signals from the T cell receptor and TLRs (Sun et al., 2008). Mice that lacked TIPE2 died from accumulation of lymphocytes and macrophages in their lungs, liver, and intestines and of inflammatory cytokines in their blood (Sun et al., 2008). Similarly, loss of function of a myeloid-specific microRNA led to overproliferation and hyperactivity of granulocytes and to spontaneous development of interstitial pneumonitis (Johnnidis et al., 2008). When oxidation or mistranslation of proteins leads to their unfolding, the endoplasmic reticulum membrane-spanning enzyme IRE1 activates a transcription factor, X box binding protein 1. Mice in which XBP1 was selectively deleted from intestinal epithelial cells

ways, such as protein and lipid phosphatases, kinase-inhibiting proteins, ubiquitin ligases, and transcriptional regulators.

Cells Contributing to Resolution

Perhaps the most important reason that host cell death without microbial involvement rarely triggers nonresolving inflammation is that the death is usually apoptotic; apoptotic cells are ingested by viable cells, typically macrophages; and ingestion of apoptotic cells triggers macrophages to release inflammation-resolving cytokines such as TGF- β and IL-10 (Huynh et al., 2002; Kennedy and DeLeo, 2009). Glucocorticoids, whose levels are often increased in response to the stress associated with an inflammatory condition, promote ingestion of apoptotic cells by macrophages and their release of TGF- β and IL-10 (Kennedy and DeLeo, 2009).

In contrast, if apoptotic cells are not ingested rapidly, they often progress to necrosis. Necrosis releases products that are agonists, or generate agonists, for host receptors that also detect microbial products and that generate proinflammatory signals. Inflammation can thus be prolonged by a failure of neutrophils to undergo timely apoptosis or by a failure of macrophages to clear those that apoptose. Neutrophil survival is

Table 1. Gene Products Required to Maintain Basal Anti-inflammatory Tone

Functional Class	Gene Product
Regulation of Apoptosis and Clearance of Apoptotic Cells	<u>Fas</u> , <u>Fas</u> , <u>C1q</u> , <u>C1q</u> , <u>C2</u> , <u>C4</u> , <u>C4</u> , <u>C3</u> , <u>C4 BP</u> , <u>factor H</u> , <u>Crry</u> , <u>SAP</u> , <u>DNAse I</u> , <u>DNAse II</u> , <u>FcγRIIB</u> , <u>WASP</u> , <u>WASP</u> , <u>c-mer</u> , <u>caspase 8</u> , <u>TIPE2</u>
Cytokines and Cell Surface Receptors	<u>TNFR1</u> , <u>TNFR1</u> , <u>TGF-β1</u> , <u>IL-2Rα</u> , <u>IL-2</u> , <u>IL-10</u> , <u>IL-10R</u> , <u>GM-CSF</u> , <u>GM-CSF</u> , <u>IL-1Ra</u> , <u>IL-1Ra</u> , <u>TcRα</u> , <u>TcR β</u> , <u>MHCII</u> , <u>CTLA4</u> , <u>PD1</u> , <u>TAC1</u> , <u>a$\epsilon$$\beta$7 (CD103)</u> , <u>$\alpha$v integrin</u>
Other Membrane or Intracellular Proteins of Lymphocytes, Leukocytes, or Epithelial Cells Affecting Their Activation	<u>ZAP70</u> , <u>MDR1α</u> , <u>LAT</u> , <u>TSAd</u> , <u>SOCS1</u> , <u>PI3K p110δ</u> , <u>PTEN</u> , <u>lyn</u> , <u>cbl-b</u> , <u>Gzi2</u> , <u>SHP-1</u> , <u>SHIP</u> , <u>p21</u> , <u>TIA-1</u> , <u>tristetraproline</u> , <u>A20</u> , <u>NFAT</u> , <u>IKK-2</u> , <u>IKKγ</u> , <u>IKKγ</u> , <u>IkBα</u> , <u>IkBβ</u> , <u>Rel-b</u> , <u>NF-κB1</u> , <u>Ndfip1</u> , <u>T-bet</u> , <u>Gadd45a</u> , <u>IRF-2</u> , <u>RabGEF1</u> , <u>NOD2/CARD15</u> , <u>pyrin</u> , <u>cryopyrin</u> , <u>mevalonate kinase</u> , <u>Foxj1</u> , <u>Foxo3a</u> , <u>p120-catenin</u> , <u>RXRα/β</u> , <u>JunB/cJun</u> , <u>TRAF6</u> , <u>CAT2</u> , <u>PSTPIP1</u> , <u>PSTPIP2</u> , <u>α-mannosidase-II</u> , <u>RUNX3</u> , <u>SH3BP2</u> , <u>TAK1</u> , <u>XBP1</u>
Other	<u>Heme oxygenase 1</u> , <u>surfactant protein D</u> , <u>STAMP2</u> , <u>miR-223</u>

Proteins encoded by genes whose mutation leads to spontaneous inflammatory states not attributable to infection or autoimmunity. Human products are underlined; others are mouse. For abbreviations and references, see Table S1. This table is updated from Nathan (2002).

prolonged in many inflammatory states by the ability of GM-CSF and G-CSF to induce the apoptosis inhibitor survivin (Altzner et al., 2004) and the ability of hypoxia inducible factor-1 α to induce glycolytic enzymes (Cramer et al., 2003) that support ATP generation under hypoxic conditions. Although complement activation is generally proinflammatory, dependence of apoptotic cell clearance on complement implies that inflammation might be prolonged in the face of a deficiency in the complement system (Mevorach et al., 1998). Likewise, inflammation might be prolonged by deficiency of other factors that promote ingestion of apoptotic cells, such as the secreted glycoprotein milk fat globule-EGF-factor 8 (MFG-E8) (Hanayama et al., 2002), or TIM4, a macrophage receptor for the phosphatidylserine exteriorized by apoptotic cells (Miyanishi et al., 2007).

Along with the contrasting impact of encountering apoptotic or necrotic cells, there are other ways in which macrophages normally switch from being proinflammatory at the outset of an inflammatory response to anti-inflammatory later in the process. For example, in wound healing, inflammatory monocytes accumulate in damaged tissue and are essential for its repair (Arnold et al., 2007). In unexplained contrast to the impact of products of necrotic cells, phagocytosis of tissue debris can induce mononuclear phagocytes to switch from a proinflammatory to an anti-inflammatory phenotype that promotes muscle cell differentiation in conjunction with production of elevated amounts of TGF- β (Arnold et al., 2007). TGF- β is a potent suppressor of what is now called classical macrophage activation (Tsunawaki et al., 1988) and a critical mediator of tissue repair. Tissue damage also promotes production of glucocorticoid hormones. These, too, can convert monocytes/macrophages from a proinflammatory to anti-inflammatory state (Ehrchen et al., 2007; Perretti and D'Acquisto, 2009). IL-4 released by mast cells, basophils, and Th2 cells provides another cue for macrophages to switch from a classically activated, proinflammatory phenotype to one that suppresses inflammation and favors wound healing, including through production of IL-10 (Mosser and Zhang, 2008). After TGF- β , IL-10 was the second cytokine produced by nontransformed cells that was discovered to be a potent suppressor of classical macrophage activation (Bogdan et al., 1991).

In some settings, a profile of cell surface markers and molecular products distinguishes macrophage populations that carry out two distinct aspects of resolution—suppression of inflammation and promotion of tissue repair (Mosser and Edwards, 2008). It is not clear whether this reflects alternative pathways of differentiation on the part of cells in the affected tissue or sequential recruitment of cells with different profiles. In the myocardium of mice after occlusion of a coronary artery, shifts over time in expression of chemokines and adhesion molecules allowed sequential recruitment of different monocyte populations. The first monocyte population contributed to the evolving inflammatory response and the second to its resolution (Nahrendorf et al., 2007). The monocytes that predominated in the damaged tissue from days 1–4 were Ly6C^{high} cells mobilized largely from the spleen in response to angiotensin II (Swirski et al., 2009). These cells entered the heart in response to signaling via CCR2, expressed high levels of matrix metalloproteinases and TNF- α , and were required for degradation of the damaged tissue

(Nahrendorf et al., 2007). From days 5 through 16, the most prevalent monocytes were Ly6C^{lo} cells responding via CX3CR1; they expressed far less matrix metalloproteinases but high levels of VEGF and contributed to angiogenesis and deposition of collagen (Nahrendorf et al., 2007).

Distinct from the macrophage populations discussed above are myeloid-derived suppressor cells, a heterogeneous group of myeloid progenitors that expand rather than differentiate in response to prostaglandins, VEGF, and other factors that activate STAT3. They can accumulate in the spleen and blood and enter cancers, infected sites, and inflamed organs in mice with inflammatory bowel disease, experimental allergic encephalomyelitis, or experimental autoimmune uveitis (Gabrilovich and Nagaraj, 2009). Through inducible nitric oxide synthase (iNOS)-dependent production of peroxynitrite and arginase-mediated consumption of L-arginine, among other means, these cells can profoundly suppress T cell responses (Gabrilovich and Nagaraj, 2009). Given that effector T cell-derived chemokines and macrophage-activating factors can contribute to progression of inflammation, suppression of T cells may help inflammation resolve. On the other hand, premature abrogation of T cell effector functions may allow inflammatory stimuli to persist. For example, suppressed T cells may fail to restrain the growth of chemokine-secreting cancer cells that prolong inflammation, further inducing the expansion and activation of myeloid-derived suppressor cells.

There are diverse additional ways in which an abnormal T cell response could lead to prolonged inflammation. T regulatory cells (see Review by D.R. Littman and A.Y. Rudensky on page 845 of this issue) can suppress inflammation (Garrett et al., 2007). A deficiency in T regulatory cells appears to contribute to the persistent inflammation of visceral adipose tissue in obesity (Lo, 2009). The T cell products IFN- γ and TNF- α are critical for halting production of macrophage-derived chemokines (Hodge-Dufour et al., 1998), and effector and memory CD4⁺ T cells can suppress macrophages' production of IL-1 β (Guarda et al., 2009). Thus, even though a Th1 response can contribute to inflammation, a subnormal Th1 response can prolong it.

Soluble Factors Contributing to Resolution

Above, we discussed the shifting nature and impact of cell populations as inflammation resolves. Also driving resolution is the secretion or extracellular formation of soluble products. Resolution may fail if expression of these factors is delayed or reduced. The examples discussed here illustrate their diversity: cytokines, a protease inhibitor, gaseous signals, oxygenated and nitrated lipids, a purine, and a neurotransmitter (Figure 4).

Among inflammation-resolving cytokines, many roads of research lead to IL-10 and TGF- β , two of the most important mediators of resolution of inflammation. Production of IL-10 generally requires two signals (Mosser and Zhang, 2008). One signal may be immune complexes, prostaglandins, adenosine, or apoptotic cells, while the other may be a TLR ligand (Mosser and Zhang, 2008). Thus, full-scale production of IL-10 normally ensues only after inflammation is likely to have developed.

Cases of Crohn's disease that are attributable to a mutant NOD2 protein illustrate the clinical significance of inadequate IL-10 production during inflammation. Truncated NOD2 blocks the ability of p38 kinase to phosphorylate a ribonucleoprotein

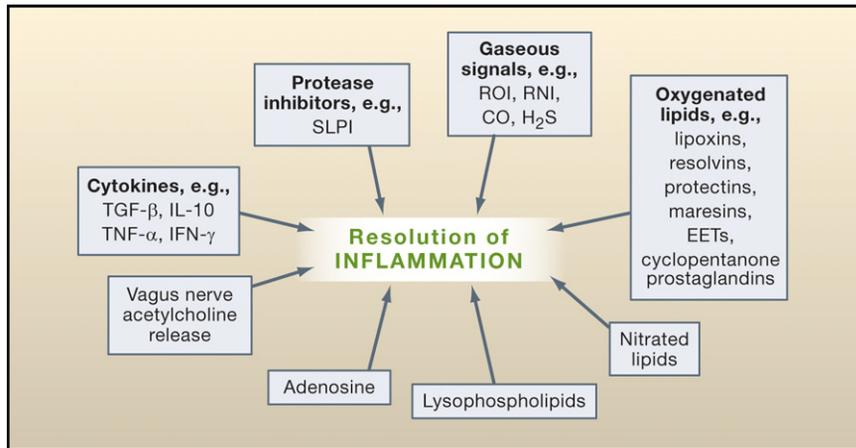


Figure 4. Examples of Mediators of Resolution that Are Secreted or Formed Extracellularly

required for transcription of IL-10 (Noguchi et al., 2009). Deficient expression of IL-10 likely contributes to the sustained inflammation. Another explanation for Crohn's disease, to be discussed subsequently, may help explain why the disease is usually localized to the bowel.

IL-10 and TGF- β are downstream points of convergence for several other interacting cytokines that play important roles in inflammatory resolution. For example, resolution of chemically-triggered colitis in mice required IL-13, which served to increase production of IL-10 and TGF- β (Fichtner-Feigl et al., 2008).

Other secreted proteins besides cytokines play controlling roles in the resolution of inflammation, raising the possibility that their subnormal release may be involved when inflammation is abnormally persistent. A striking example is secretory leukocyte protease inhibitor (SLPI). SLPI is secreted late in the response of macrophages to inflammatory stimuli such as LPS (Jin et al., 1997). SLPI suppresses the ability of TNF- α to activate neutrophils. SLPI also binds to the epithelial growth factor progranulin and protects it from digestion by elastase, an enzyme normally released by activated neutrophils in response to TNF- α . When digested by elastase, progranulin gives rise to granulins, polypeptides that trigger epithelial cells to release the neutrophil-attracting chemokine IL-8. When protected by SLPI, the intact progranulin molecule instead promotes epithelial repair without chemokine production (Zhu et al., 2002). The SLPI-progranulin axis is critical for resolution of inflammation in wounded skin and for prompt closure of the wound (Zhu et al., 2002; He et al., 2003).

Small, covalently reactive molecules such as ROI and reactive nitrogen intermediates (RNI) are important anti-inflammatory factors, although they are better known for proinflammatory effects. These diffusible molecules display atomic rather than molecular specificity and thereby integrate a wide range of signaling reactions (Nathan, 2003). Pro- and anti-inflammatory actions of ROI will be discussed below in connection with excessive and subnormal responses, respectively. Here, we comment briefly on RNI arising from NO, the primary product of iNOS (NOS2), NOS1, and NOS3. Proinflammatory actions of RNI cause lethal pneumonitis in mice infected with influenza virus (Karupiah et al., 1998). One key mechanism may be that iNOS binds, nitrosylates, and activates cyclooxygenase-2 (COX2)

The IL-10- and stress-induced enzyme heme oxygenase-1 produces bilirubin (an antioxidant) and carbon monoxide (CO). CO not only mediates some of the anti-inflammatory effects of IL-10 (Lee and Chau, 2002), but also enhances IL-10 expression and suppresses LPS-induced production of TNF- α (Otterbein et al., 2000). Another gaseous mediator, hydrogen sulfide (H₂S), can increase the production of CO. H₂S is generated from cysteine by cystathionine- γ -lyase and cystathionine- β -synthase (Szabó, 2007). H₂S can exert anti-inflammatory effects in inflammation awaits studies with the recently produced knockout mice for the H₂S-producing enzymes (Mustafa et al., 2009) and better understanding of how its production is regulated.

Diverse classes of oxygenated and nitrated lipids contribute to the resolution of inflammation. In experimental inflammatory exudates, the early oxygenation of arachidonic acid (20:4) into inflammatory prostaglandins and leukotrienes is superseded by conversion of arachidonate or 15-hydroxyeicosatetraenoic acid into lipoxins. Lipoxins can suppress neutrophil chemotaxis, vascular dilatation, vascular permeability, fibrosis, and pain, while promoting emigration of monocytes and their ingestion of apoptotic neutrophils (Serhan, 2007). Also generated in the resolving phase are eicosapentaenoic acid (20:5)-derived resolvins and docosahexaenoic acid (22:6)-derived protectins (Serhan, 2007) and maresins, the latter dependent on a 14-lipoxygenase (Serhan et al., 2009). The shifting profile of oxygenated lipids reflects both the temporal expression of different enzymes and the mobilization of different fatty acid substrates for use by some of the same enzymes. Early events entrain later ones; for example, prostaglandin E2 and prostaglandin D2 induce neutrophils to express 15-lipoxygenase, launching the production of lipoxins (Serhan, 2007).

Arachidonic acid-derived epoxyeicosatrienoic acids (EETs) produced by cytochrome P450 epoxygenases represent yet another class of anti-inflammatory lipids. EETs can inhibit NF- κ B and block induction of COX2 and 5'-lipoxygenase (Node et al., 1999).

COX2 has also been found to switch from producing the predominantly proinflammatory PGE2 early in inflammation to producing cyclopentanone prostaglandins later on, such as

15-deoxy- $\Delta^{12,14}$ -prostaglandin J. The latter blocks endothelium from expressing VCAM-1, macrophages from releasing chemokines, and adherent neutrophils from producing ROI, among other anti-inflammatory actions (Lawrence et al., 2002). Thus, while administration of COX2 inhibitors early is anti-inflammatory, their late administration can prolong inflammation (Gilroy et al., 1999), and there is a similar concern with 5'-lipoxygenase inhibitors (Serhan, 2007). This may help explain the failure of COX2 inhibitors to limit brain damage in stroke patients.

Nitrated fatty acids are abundant in body fluids. They have a potent ability to inhibit NF- κ B, neutrophil activation, and expression of iNOS and VCAM-1; to activate peroxisome proliferator-activated receptor- γ (PPAR γ); and to induce heme oxygenase-1 (Freeman et al., 2008). Full appreciation of the role of nitro-fatty acids awaits better understanding of how their levels are regulated during adaptive and maladaptive responses.

Lysophospholipids may also play an anti-inflammatory role. For example, sphingosine-1-phosphate (S1P) bound to high-density lipoproteins helps stimulate endothelial cells to release NO (Nofer et al., 2004). NO arising in endothelium helps prevent adherence of platelets and neutrophils. Endothelial cell S1P receptor 3 (S1P3) may be particularly important in mediating the tonic antipermeability effect of S1P in plasma. The diverse actions of the five known S1P receptors on different cell types, limited knowledge of S1P gradients in inflammatory states, and the ability of some chemical probes considered antagonists to behave as partial agonists contribute to the difficulty in evaluating the anti-inflammatory role of S1P.

Additional autacoids are critical in restraining inflammation and may be important in its resolution. For example, necrotic cells release ATP, which is rapidly broken down by ectonucleotidases, producing adenosine. Proinflammatory actions of adenosine were noted above. However, the A2a adenosine receptor is critical to suppress inflammation (Ohta and Sitkovsky, 2001), and its agonists can promote wound healing (Haskó and Cronstein, 2004).

Tracey and colleagues have illuminated the existence of a powerful anti-inflammatory pathway mediated by the vagus nerve via its innervation of the viscera, particularly the spleen and liver. Vagus nerve terminals release acetylcholine, which acts on α 7 nicotinic cholinergic receptors to suppress release of TNF- α , IL-1 β , IL-6, IL-8, and HMGB1 by macrophages in those organs. Because this pathway is activated in the brain in response to infection, injury, ischemia, and inflammatory cytokines, it constitutes an "inflammatory reflex" (Tracey, 2007). Electrical stimulation of the vagus or administration of cholinergic agonists can exert a therapeutic effect in inflammatory states (Tracey, 2007).

In sum, late in an inflammatory reaction, potent molecules that signal via specific receptors to block inflammatory responses are synthesized or released. This demonstrates that resolution of inflammation is an active process, not merely what happens when proinflammatory stimuli cease (Serhan, 2007).

Persistent Stimulation

The most straightforward cause of nonresolving inflammation is the persistence of inflammatory stimuli of exogenous origin. Persistent infections by *M. tuberculosis*, *H. pylori*, schistosomes, and hepatitis viruses have been mentioned. Nondegradable

particles of asbestos and silica that trigger the inflammasome (Dostert et al., 2008) promote chronic inflammation. However, there are diverse types of persistent stimuli that are neither infectious nor particulate. Some of these arise from what we breathe or eat.

Exogenous lipid-binding proteins that are persistently present in some environments, such as Derp2 from the feces of the dust mite, can augment the host's response to microbes, promoting allergic responses. They do so by mimicking the function of MD2, the LPS-binding chain of the TLR4 receptor complex (Trompette et al., 2009). The allergic response is a form of inflammation that can become chronic, even leading to fibrotic changes (Galli et al., 2008).

Another form of persistent stimulation of inflammation arises from the biosynthetic incorporation of a foreign food antigen into self-molecules, with which antibodies then react. For example, humans have a loss-of-function mutation in a gene encoding an enzyme required for production of N-glycolylneuraminic acid (Neu5Gc), which the human immune system can therefore recognize as foreign (Hedlund et al., 2008). Dietary Neu5Gc is taken up from red meat and dairy products, incorporated into cell surface glycans, and bound by antibody. The resulting inflammation appears to promote angiogenesis and oncogenesis in a COX2-dependent manner (Hedlund et al., 2008).

Some chronic inflammatory diseases appear to begin with repeated exposure to a toxin, leading to tissue injury that provokes an autoimmune reaction. The autoimmune reaction may then perpetuate the inflammation. This is an emerging view of chronic obstructive pulmonary disease, the fourth leading cause of death in many industrialized countries. Cigarette smoke is a major precipitant. In mice, the ability of cigarette smoke to inflame the lungs requires TLR4 and MyD88. This suggests a reaction to products of dead host cells or damaged extracellular matrix. Cigarette smoke also impairs the ability of macrophages to ingest apoptotic cells. Large numbers of activated, oligoclonal CD4+, and CD8+ T cells accumulate in the lungs, along with B cell-rich lymphoid follicles (Cosio et al., 2009). Similarly, recent evidence raises the possibility that obesity may be propagated in part by an autoimmune reaction to an antigen arising in adipose tissue. Th1 cells promoted obesity-associated insulin resistance and weight gain in mice. T cells in visceral adipose tissue had a biased antigen receptor repertoire, suggesting that they had undergone local antigen-driven expansion (Lo, 2009). The Th1 cells may activate macrophages in adipose tissue, as discussed in the next section.

Cancers that thrive on the angiogenic products of inflammatory cells are another cause of persistent proinflammatory stimulation. Tumors court inflammatory cells by generating chemokines (Mantovani et al., 2008) and can activate them by producing matrix proteins like versican that bind to the TLR2-TLR6 heterodimer (Kim et al., 2009).

Nonresolving Inflammation Linked to a Prolonged or Excessive Response

Any molecular driver of inflammation can probably delay resolution of inflammation if its production goes on too long or at too high a level. Here, we will illustrate this for one driver, ROI. ROI are sometimes overlooked in discussions of the control of

biologic processes because their effects are so widespread that they can be misconstrued as nonspecific (Nathan, 2003). First, we list examples of specific proinflammatory effects of ROI that are new and striking. Then we illustrate contributions of ROI to several major disorders in which non-resolving inflammation plays an important role.

A novel imaging method in the zebrafish has revealed that a NOX-derived gradient of H₂O₂ develops within the first 3 min after wounding and attracts leukocytes to the injury (Niethammer et al., 2009). It seems likely that this H₂O₂-based chemoattractant mechanism operates in mammals as well. Also very early after injury, ROI may activate the transient receptor potential ion channel TRPA1 in chemosensory nerves, with proinflammatory consequences (Caceres et al., 2009). When phagocytes arrive, their NOX2 (phagocyte oxidase or phox) can activate the inflammasome to drive production of IL-1 β (Dostert et al., 2008). Later on, in an inflammatory disease like influenza in mice, NOX2 can contribute to the death of the host (Imai et al., 2008), apparently by synergizing with RNI (Karupiah et al., 1998) to produce peroxynitrite (Akaike et al., 1996).

Excess production of ROI plays a prominent role in the pathogenesis of most if not of all the major diseases of nonresolving inflammation. Mechanisms that get the lion's share of attention differ in each case. For example, in atherosclerosis (Galkina and Ley, 2009), excess superoxide diverts endothelial-derived NO from its anti-inflammatory and antihypertensive role into the formation of peroxynitrite. Peroxynitrite damages mitochondria and promotes more superoxide formation. In chronic obstructive pulmonary disease (Cosio et al., 2009), oxidative inactivation of α 1-anti-trypsin, α 2-macroglobulin, and SLPI contributes to breakdown of elastic tissue.

Obesity is the form of nonresolving inflammation whose incidence is rising the fastest. Thus, obesity serves here as a focus for illustrating the role of excess ROI in a bit more detail. For primary references, please see Nathan (2008). Excessive caloric intake is associated with excessive production of ROI in adipocytes, vascular smooth muscle, and endothelial cells. The ROI arise from three intracellular sources—the mitochondrial electron transport chain, the endoplasmic reticulum, and NOXs. In hypertrophied adipocytes, the large lipid vacuole stores more oxygen than an equivalent volume of cytosol. The engorged adipocytes compress blood vessels in the fat, making themselves hypoxic. Hypoxia promotes mitochondrial dysfunction and leakage of electrons into ROI generation, which the lipidic oxygen reservoir may sustain. The tiny cytosolic compartment of the hypertrophied adipocyte contains a subnormal quantity of antioxidant systems, and, on a per-molecule basis, they are subnormally active. Thus, the hypertrophied adipocyte may have a markedly adverse balance between oxidant production and antioxidant defense. Excess ROI further disrupt mitochondrial electron transport and promote their own formation. ROI can drive the production of a 4-OH-*trans*-2-nonenal, a lipid aldehyde that activates the proinflammatory ion channel TRPA1. The lipid aldehyde also reacts with glutathione. Aldose reductase acts on the adduct, generating glutathionyl-1,4-dihydroxynonane, which can induce TNF- α , IL-1 β , IL-6, and chemokines in macrophages (Yadav et al., 2009). Macrophages accumulate, adipocytes necrose, and more macrophages

accumulate. Inflammatory cytokines arising in visceral adipose tissue (see Review by G.S. Hotamisligil on page 900 of this issue) circulate and adversely affect other tissues, including the vasculature and the brain, where appetite regulation is further disordered (Nathan, 2008).

Nonresolving Inflammation Linked to a Subnormal Response

Paradoxically, a subnormal inflammatory response can engender a prolonged response. This was perhaps first illustrated by the exaggerated and ultimately lethal inflammatory response of TNF- α ^{-/-} mice to injection of *Propionibacterium acnes* (then called *Corynebacterium parvum*) (Hodge-Dufour et al., 1998). Here, we consider three recent examples that illustrate three additional mechanisms: Crohn's disease, whose genetic basis is unknown in most cases; chronic granulomatous disease (CGD), caused by genetic deficiency in NOX2; and influenza A virus infection in mice with an engineered deficiency in the generation of IL1- β and IL-18.

Work over three decades by Anthony Segal and colleagues, as recently extended (Smith et al., 2009), suggests that a fundamental defect in macrophages from Crohn's disease patients (with or without NOD2 mutations) consists in the misdirection of certain cytokines from the secretory pathway to lysosomal degradation (Smith et al., 2009). The failure of macrophages to release neutrophil-attracting cytokines when challenged with large numbers of *E. coli* may lead to inadequate neutrophil recruitment at sites of bacterial penetration of the intestinal mucosa, resulting in a stronger inflammatory stimulus at a deeper site than would be found in healthy subjects. In this view, what would normally have been no inflammation or acute, resolving, subclinical inflammation becomes recurrent, chronic, and pathologic.

In CGD, a defect in the respiratory burst of neutrophils, monocytes, and macrophages predisposes to recurrent, persistent infection, and granulomatous inflammation. However, in both Crohn's disease and CGD, the relationship between infection and nonresolving inflammation is likely to be complex, since the granulomas (nodular accumulations of phagocytes and lymphocytes, with or without a fibrous rim) are often found to be microbiologically sterile. This could result from infection by microorganisms we do not know how to detect, as was the case until recently for *Granulibacter bethesdaensis* in some patients with CGD. However, it is also clear that deficient ROI production can initiate or contribute to chronic inflammation in the absence of infection. In a mouse model of CGD, this was demonstrated by a nonresolving inflammatory response to nonviable microbial products (Morgenstern et al., 1997).

Mechanisms for the anti-inflammatory role of ROI are beginning to be identified. ROI activate the transcription factor Nrf2. Nrf2-dependent upregulation of enzymes that synthesize the antioxidant tripeptide glutathione is essential for resolution of inflammation and repair of damaged tissue (Reddy et al., 2009). ROI oxidize cholesterol to a form that activates liver X receptors (LXRs), whose heterodimerization with retinoid X receptor (RXR) leads to inhibition of expression of COX2, iNOS, and IL-6 (Joseph et al., 2003). NOX2-generated superoxide is a cofactor for indoleamine dioxygenase, whose product, kynurenine, is essential for the mouse to restrain what is

otherwise excessive IL-17 production and inflammation (Romani et al., 2008).

Finally, mice lacking NLRP3 or caspase 1 were deficient in production of IL-1 β and IL-18 in response to influenza A virus, were slower to recruit neutrophils and monocytes to the lung, and produced lower amounts of TNF- α and IL-6 than the wild-type (Thomas et al., 2009). Even though these mice controlled the virus as readily as did wild-type mice, they paradoxically suffered more epithelial necrosis, fibrosis, and mortality (Thomas et al., 2009).

Challenges for Biology

There is now an enormous literature on nonresolving inflammation. However, understanding of the principles has not reached a level that permits accurate predictions. Here are some key challenges.

We have argued that clinically evident inflammation usually begins in response to signals of two types, one type reporting injury and the other infection, or in response to extensive injury alone, which sensors for host cell necrosis interpret as being accompanied by infection. In contrast, when chronic, nonresolving infection lacks an identifiable, persistent, exogenous stimulus, it is not clear what signals sustain it. How widespread is the role of the oxidized self-lipid that activates TLR4 (Imai et al., 2008)? Does inflammation itself sometimes reset thresholds so that receptors become sensitive to levels of proinflammatory signals that are normally ignored? Do chromatin modifications prolong responses to signals that have subsided?

We have stressed that cells like macrophages and T cells and molecules like TNF and ROI can exert both pro- and anti-inflammatory effects. We also emphasized the heterogeneity of pathologic forms of chronic inflammation within an affected organ. Does it follow that the same cells or signals can have different effects in different microenvironments of the same organ at the same time?

The impact of a signal depends on the other signals present as well as their history (Nathan and Sporn, 1991). Thus, it is not surprising that the same intervention may produce opposite effects at different times or in different subjects. What information do we need to predict the effect of blocking a given signal in a given inflammatory disease at a given point in its progression? Does the state of the extracellular matrix offer a useful record of a tissue's history and a guide to its response (Nathan and Sporn, 1991)?

Another theme has been the pervasive influence of ROI and RNI in promoting and resolving inflammation. Perhaps the only other class of signaling mediators with such a wide influence on inflammation is the nuclear receptor superfamily. Many drugs regulate nuclear receptors, but few effectively regulate ROI or RNI. Can biology now guide the development of a clinically effective antioxidant pharmacology? Is it possible to develop selective inhibitors of specific sources of ROI and RNI, such as electron-leaking mitochondria, stressed endoplasmic reticulum, uncoupled NOSs, and individual NOXs? Could some such inhibitors work by compensating for or reversing damage to mitochondria, endoplasmic reticulum, or NOSs?

We offered two illustrations of the concept that localized sites may continue to drive a chronic inflammatory disorder that has

become systemic. Thus, we speculated that generation of citrullinated proteins by peptidyl arginine deiminase from dying neutrophils in rheumatoid joints may perpetuate the formation of antigen-antibody complexes. We referred to evidence that hypertrophic, macrophage-rich visceral fat generates cytokines that affect not only the pancreas, vasculature, liver, and skeletal muscle, but also the brain, dysregulating appetite control and helping perpetuate obesity. Is there a biologic basis by which to target potent anti-inflammatory therapies to localized sites, minimizing the risks and toxicities of systemic action? For example, can we exploit the differential expression of adhesion receptors for this purpose or use inflammatory environments or enzymes to activate prodrugs?

We have emphasized the molecular diversity of inflammation-resolving signals. It is practical and customary to study them one at a time. To our knowledge, there has been no study of a chronic inflammatory state in which all known resolution factors—cytokines, protease inhibitors, gaseous signals, oxygenated and nitrated lipids, lysophospholipids, autacoids, neurotransmitters, and so on—have been measured at the same time and over time. The potential value of a comprehensive evaluation seems clear, even at the risk of spawning a term like “resolvome.”

Implications for Therapy

The biologic complexity of nonresolving inflammation and our limited understanding of it combine to make the development of anti-inflammatory therapy particularly difficult. Moreover, the most potent anti-inflammatory interventions have the potential to increase the risk of infection, and every anti-inflammatory therapy now in use has one or more potentially serious toxicities of another kind.

Nonetheless, we should try to meet a challenge even more demanding than suppressing nonresolving inflammation. The goal should be to cure, not to palliate, inflammatory diseases. For all the success of disease-modifying antirheumatic drugs and biologics, for example, few patients with rheumatoid arthritis achieve lasting remission after the cessation of therapy. In fact, to our knowledge, no anti-inflammatory therapy cures the majority of patients with a disease in which inflammation plays a major contributory role, where “cure” means that the patient returns to good health, or at least incurs no further tissue damage, without ongoing treatment. Nor have medical treatments that are focused on pathogenic factors other than inflammation regularly cured any of the major, noninfectious, inflammation-associated diseases discussed in this review.

When dealing with major, chronic diseases of high morbidity in which inflammation plays a prominent part, we are likely to need multi-pronged therapeutic approaches. An anti-inflammatory component of therapy might synergize with components that target other causal factors, particularly if it interrupts positive feedback loops that help drive the disease (Nathan, 2008).

For an integrated therapeutic strategy to succeed, new kinds of team approaches may be required, both in academia and in industry. In academia, the biology of the cellular and soluble factors that contribute to inflammation has traditionally been the province of immunologists, pharmacologists, pathologists, and hematologists. Biochemists, molecular biologists, microbiologists, nutritionists, and endocrinologists have generally

studied metabolism; cell biologists, pathologists, and cardiologists have studied atherosclerosis; and immunologists and pulmonologists have studied asthma. Inflammation and metabolism are highly interconnected, and each of these diseases has much to teach those who study the others.

In the pharmaceutical industry, managers used to hand off drug development projects from one team of specialists to another in linear fashion, for example, from cell biologists or immunologists to structural biologists to medicinal chemists to pharmacologists and toxicologists and then to clinicians. Many companies now convene teams in which all these disciplines are represented at once so that interactions are multilateral and iterative. This is a major advance, but even then, anti-inflammatory drug discovery is often relegated to one team that works separately from others devoted to atherosclerosis, obesity, asthma, chronic obstructive pulmonary disease, neurologic disease, or cancer. Each team seeks to develop compounds or biologics that show individual promise to meet that team's goals. Therapeutic strategies that involve combined interventions, in which each intervention (a chemical, a biologic, or a behavior) is designed from the outset to complement the others, are almost never considered. Development of integrated therapeutic strategies will require building other kinds of teams.

Integrated therapeutic strategies should embrace the concept that each intervention targets at least one pathway, not just a molecule. The history of anti-inflammatory therapy is largely the story of occasional, empirical success in modifying what turn out to be control points on suitable pathways. In fact, early anti-inflammatory agents were deployed long before their targets were known. The targets proved to be enzymes (as for aspirin and similarly acting nonsteroidal anti-inflammatory agents) or transcription-regulating factors (as for glucocorticoids), and in some cases remain uncertain (as for gold salts). Statins are among the highest-impact anti-inflammatory agents, and their target, hydroxymethylglutaryl CoA reductase, was not anticipated to have anything to do with inflammation. More recently, successful anti-inflammatory agents were introduced whose targets were identified in advance, such as cytokines (TNF- α or IL-1 β), receptors (for cysteinyl leukotrienes), or adhesion molecules ($\alpha 4$ integrin). However, anti-inflammatories that are clinically useful and acceptably safe are a fraction of those whose development was undertaken.

Can we identify suitable pathways and control points by approaches more efficient than trial and error? Can we predict which individuals are more likely to benefit from a given intervention and which more likely to suffer adverse effects? These questions pertain to the biology, but there is also a need to reduce empiricism and increase efficiency in the ensuing medicinal chemistry. As each lead compound is developed, vast quantities of information are generated but then hidden away rather than used to advance collective understanding. Information sharing coupled with innovative statistical approaches could provide a breakthrough for both target identification and pharmaceutical development.

For target identification, the "genetics of genomics" (Chen et al., 2008; Emilsson et al., 2008) holds promise to reveal disease networks in terms of their edges and nodes. For the anti-inflammatory component of a therapeutic strategy, we

need to understand what nodes have such connections and such a balance of nonredundancy and redundancy that their complete or partial interruption (or in some cases, enhancement) can afford anti-inflammatory efficacy with a predictable and acceptable level of risk. Thinking in terms of pathways will encourage us to explore not only new targets in classes that have provided historical success, but also targets in new classes, such as channels that gate ions, enzymes that modify chromatin, and small RNAs that control large ones.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table and can be found with this article online at [doi:10.1016/j.cell.2010.02.029](https://doi.org/10.1016/j.cell.2010.02.029).

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